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THE CHALLENGE OF CO-EXISTENCE: FROM GRAFT-VERSUS-HOST DISEASE TO STABLE MIXED CHIMERISM AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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ABSTRACT

The only curative treatment strategy for many hematologic and inborn immunodeficiency disorders is an allogeneic hematopoietic stem cell transplantation (HSCT). The treatment involves replacing the entire hematopoietic system of the recipient. If successful, the underlying condition of the patient is resolved, the donor hematopoietic system engrafts and a tolerance between donor- and patient-derived cells is developed. Though the procedure of HSCT has been refined for decades, there are still several severe complications associated to it.

Graft-versus-host disease (GVHD) is one of the most common and most feared complications post-HSCT, and is a result of donor graft-derived cells attacking recipient tissue. Despite improved GVHD treatment strategies, severe grade GVHD is still associated with high morbidity and mortality rates. A condition known as mixed chimerism (MC), where recipient hematopoietic cells co-exist with donor hematopoietic cells, may also be considered an adverse event early post-HSCT. This is certainly the case for patients with malignancies as it indicates a potential relapse. However, in rare cases where HSCT is performed due to non-malignant disorders, long-term stable MC may develop without any apparent signs of unfavourable symptoms.

The papers in this thesis aim to provide a better understanding of the co-existence of graft- and host-derived cells from an immunological perspective. I will focus on GVHD and long-term stable MC post-HSCT particularly.

In **Paper I**, I aimed to identify predictive markers for acute GVHD development. Acute GVHD occurs relatively shortly post-HSCT with potential devastating effects. In this paper, I observed a reduced frequency in mucosal-associated-invariant T (MAIT) cells in donor grafts, given to patients who later developed acute GVHD. Moreover, I could identify a predictive role for high PD-1 and low CD127-expressing T cell frequencies in the donor grafts. Together with increased levels of TNFα in both the donor graft and in patient plasma prior to HSCT, these findings may putatively be used to predict acute GVHD development in patients at time of transplantation.

In **Paper II**, I focused on chronic GVHD, a complication that usually develops later post-HSCT presenting with symptoms from several organs. Patients may suffer from chronic GVHD for years, resulting in a diminished quality of life. In this paper, I was able to identify novel cellular subsets via mass cytometry that could be linked to the severity of chronic GVHD. These subsets could also be identified by conventional flow cytometry panels more suitable for routine laboratories. Additionally, similar to the study on acute GVHD, patients with more pronounced chronic GVHD were found to have fewer MAIT cells in their blood. Thus, **Paper I** and **II** indicate a potential role for MAIT cells in both acute and chronic GVHD.

In **Paper III** and **IV**, the focus was long-term stable MC, which is defined as the presence of 5-95% recipient-derived cells, after ≥5 years post-HSCT in this study. Interestingly, patients with long-term stable MC had a similar quality of life, infectious disease burden and response to vaccines compared to patients with full donor chimerism (DC). Fluctuations in recipient chimerism tended to decrease and reach stable levels after around two to five years post-HSCT. Moreover, patients with MC appear to retain functional recipient-derived cells in multiple cellular subsets. Patients with MC also displayed increased levels of IgG3 and reduced lymphocyte expression of ZAP-70, though they were found to be similar to patients with DC in overall immune phenotype.

LIST OF SCIENTIFIC PAPERS

I. **Stikvoort A**, Gaballa A, Solders M, Nederlof I, Önfelt B, Sundberg B, Remberger M, Sundin M, Mattsson J, Uhlin M. Risk factors for severe acute graft-versus-host disease in donor graft composition. *Submitted manuscript* 2017

II. **Stikvoort A**, Chen Y, Rådestad E, Törlén J, Lakshimikanth T, Björklund A, Mikes J, Achour A, Gertow J, Sundberg B, Remberger M, Sundin M, Mattsson J, Brodin P, Uhlin M. Combining flow and mass cytometry in the search for biomarkers in chronic graft-versus-host-disease. *Front Immunol* 2017; **8**:717

III. **Stikvoort A**, Gertow J, Sundin M, Remberger M, Mattsson J, Uhlin M. Chimerism patterns of long-term stable mixed chimeras post hematopoietic stem cell transplantation in patients with nonmalignant diseases: follow-up of long-term stable mixed chimerism patients. *Biol Blood Marrow Transplant* 2013; **19**(5)**:** 838-844.

IV. **Stikvoort A**, Sundin M, Uzunel M, Gertow J, Sundberg B, Schaffer M, Mattsson J, Uhlin M. Long-term stable mixed chimerism after hematopoietic stem cell transplantation in patients with non-malignant disease, shall we be tolerant? *PloS One* 2016; **11**(5)**:** e0154737.

OTHER PUBLICATIONS

I. Gertow J, **Stikvoort A,** Watz E, Mattsson J, Uhlin M. Mixed chimerism after allogeneic stem cell transplantation – focus on double cord blood transplantation. *J Blood Disord Transfus* 2012; S1:006.

II. Norström MM, Rådestad E, **Stikvoort A**, Egevad L, Bergqvist M, Henningsohn L, Mattsson J, Levitsky V, Uhlin M. Novel method to characterize immune cells from human prostate tissue. *Prostate* 2014; **74**(14)**:** 1391-1399.

III. Gaballa A, Sundin M, **Stikvoort A**, Abumaree M, Uzunel M, Sairafi D, Uhlin M. T cell receptor excision circle (TREC) monitoring after allogeneic stem cell transplantation; a predictive marker for complications and clinical outcome. *Int J Mol Sci* 2016; **17**(10).

IV. Sairafi D[*], **Stikvoort A**[*], Gertow J, Mattsson J, Uhlin M. Donor cell composition and reactivity predict risk of acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *J Immunol Res* 2016; **2016**:5601204

V. Erkers T, Solders M, Verleng L, Bergström C, **Stikvoort A**, Rane L, Nava S, Ringden O, Kaipe H. Placenta-derived decidual stromal cells alter IL-2R expression and signaling in alloantigen-activated T cells. *J Leukoc Biol* 2017; **101**(3):626-632

[*] shared first authorship

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LIST OF ABBREVIATIONS

aGVHD	Acute graft-versus-host disease
APC	Antigen presenting cell
ATG	Anti-thymocyte globuline
AUC	Area under the curve
BAFF	B-cell activating factor
BCR	B cell receptor
BM	Bone marrow
BSI	Blood stream infections
Bu	Busulphan
CB	Umbilical cord blood
CCR	Chemokine receptor
CD	Cluster of differentiation
cGVHD	Chronic graft-versus-host disease
CLL	Chronic lymphoid leukaemia
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
Cy	Cyclophosphamide
DC	Full donor chimerism
DLI	Donor lymphocyte infusion
ELISA	Enzyme-linked immunosorbent assay
Flu	Fludarabine
G-CSF	Granulocyte colony-stimulating factor
GI	Gastro-intestinal
GVHD	Graft-versus-host disease
GVT	Graft-versus-tumour
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T
ITAM	Immune-receptor tyrosine-based activation motif
LCK	Lymphocyte-specific protein tyrosine kinase
MAC	Myeloablative conditioning
MAIT	Mucosal-associated-invariant T
MC	Mixed chimerism
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
NK	Natural killer
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PHA	Phytohemagglutinin A
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PID	Primary immunodeficiency
PMA	Phorbol 12-myristate 13-acetate
RIC	Reduced intensity conditioning
ROC	Receiver operating characteristic
TBI	Total body irradiation
TCR	T cell receptor complex
Tfh	Follicular helper T
Th	Helper T
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor-1
Treg	Regulatory T
UPN	Unique patient number
VZV	Varicella zoster virus
ZAP-70	Zeta-chain-associated protein kinase 70

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

Our bodies are under daily attack by pathogens, organisms that cause harm or disease. Pathogens range from viruses, bacteria to parasites. To combat them, an intricate defence system called the immune system is active round-the-clock. The immune system consists of an extensive network of cells present in (almost) all parts of our body, and has the ability to communicate and develop appropriate defence strategies specific to the type of invading pathogen. The importance of this intricate system becomes evident when we consider patients with immune deficiency disorders, who may suffer from lethal infections. Additionally, from an evolutionary standpoint, the immune system has proven to be an essential part of life. We can observe similar systems in other animals and even in plants, albeit in a more rudimentary form in the case of the latter.[1, 2] This indicates the necessity and importance of immune systems and that they have co-evolved with us and the pathogens trying to invade.

The immune system can be divided into two general "arms"; the innate and the adaptive. The innate arm is considered more evolutionary conserved. Versions similar to the innate system have been observed in most animals, both in invertebrates and vertebrates. Variations of the adaptive system, on the other hand, are only seen in vertebrates. Particularly so in the jawed-vertebrates, which includes fish, amphibians, reptiles, birds and mammals.[2, 3]

Innate immunity is considered to be our first line of defence. As such, its responsibilities are to; first of all, keep pathogens out; secondly, to kill pathogens that do manage to get through; and finally, to raise the alarm if the pathogens cannot be quickly destroyed. In order to do this, it needs to be able to respond fast. Hence, the innate system acts within seconds to hours after an infection. It is, however, restricted in its ability to learn. Adaptive immunity takes several days to weeks to be activated, but it can adapt its response to the pathogen and can learn from previous encounters and thus improve.

A large variety of immune cells form our immune system. All immune cells derive from a common progenitor, the hematopoietic stem cell (Figure 1). The different immune cell types will be discussed in some detail in the relevant sections. The development of a stem cell to an immune cell can be categorized in two distinct lineages, lymphoid and myeloid. The myeloid lineage ultimately forms the innate arm, while the lymphoid lineage primarily forms the adaptive arm, though not exclusively. This process of formation of all blood cells (including erythrocytes, thrombocytes and all immune cells) is called haematopoiesis. It is continuously occurring throughout human life, and is essential to a healthy immune system function and its continuous renewal.

This introduction will only touch upon some of the basics of the immune system. For a more comprehensive discussion of the immune system I would like to refer to two excellent textbooks on this matter; Parham's "The Immune System" and Janeway's "Immunobiology".[4, 5]

1.1.1 Innate Immunity

Innate immunity is fast-acting. Some of its components react within seconds to a pathogen and others may take minutes to hours. It can react so quickly as its parts are continuously patrolling and/or present. For instance, physical barriers, like the skin and mucosa, deter pathogens from entering our bodies. These barriers are considered to be an important part of innate immunity. Another example is macrophages, which continuously patrol directly below our skin, and may react within minutes to a pathogen. To understand how the innate immune system operates, it is perhaps easiest to illustrate by following a daring pathogen on its quest to invade an unsuspecting human. We will thus see how the innate immune system deals with the pathogen.

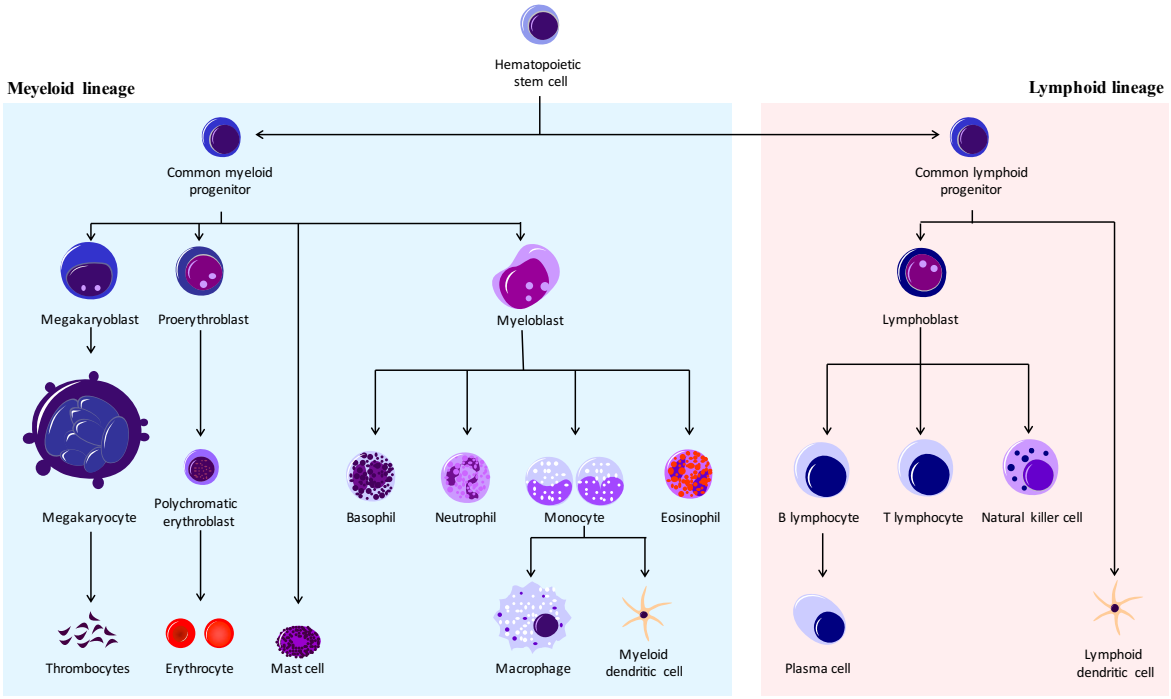

Figure 1. Schematic of haematopoiesis in humans, the development from a hematopoietic stem cell to the most common blood cells.

As mentioned, the first barrier a pathogen will need to overcome is the skin or the mucosa. The skin is protected by several layers of epithelial cells, a colony of commensal bacteria and fungi and antimicrobial peptides. Similarly, mucosal layers consist of layers of mucus and/or colonies of bacteria limiting the ability of the pathogen to attach and proliferate.[6] Therefore, in order to get past these barriers, a pathogen will need to exploit a physical disruption. This can vary from a cut, a reduced layer of mucus or a lack of commensal bacteria (for instance after an intensive antibiotic treatment). For our story's purpose, we will assume our pathogen has managed to gain entrance via a cut on a finger.

The presence of the cut is detected by the body, as damaged cells in the area send out warning signals; chemokines, cytokines and other soluble factors.[4, 5, 7] Cytokines and chemokines are small soluble proteins or protein-fragments essential for cellular communication, both short and long distance. They can affect the actions of cells around them and, as such, play a vital role in both the innate and adaptive immunity. Additionally, the pathogen itself might excrete toxins, which can also act as warning signals.[4, 5] Resident macrophages that patrol the deeper layers of the skin are attracted by the signals and will move towards the injury. There they will come into contact with the pathogen and the damaged and dead cells. They will clean up the site, a process known as phagocytosis (Figure 2) to remove the pathogen and destroyed tissue.[8, 9] Epithelial cells of blood vessels

in the area also react to the warning signals and become more porous leading to an influx of fluid into the affected area. The fluid carries platelets or thrombocytes to the site of injury which will clot the cut and prevent more pathogens from entering the body. Additionally, immune cells from the blood that are attracted by the chemokines and cytokines will migrate towards the injury. An example of such immune cells that arrive from the blood to the site of infection, are neutrophil granulocytes or neutrophils. Neutrophils are able to engulf and destroy large quantities of free roaming pathogens, which they will continue to do until they die. The dead neutrophils combined with dead pathogens form pus, which can ooze from a particularly nasty wound.[4, 5]
This bodily response to the cut and the pathogen is called an inflammatory response. It is

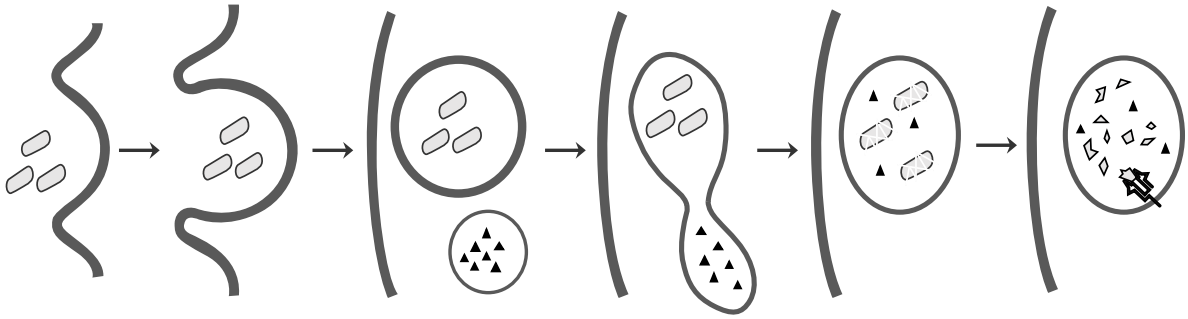

Figure 2. A representation of phagocytosis. Bacteria are engulfed by the cell into a phagosome. The phagosome fuses together with a lysosome, which contains a variety of enzymes. The enzymes destroy the bacteria into smaller proteins, which may be easily disposed of or loaded onto receptors to display on the surface of the cell.

characterized by local pain, redness of skin, swelling and warmth of the tissue around the injury. To clarify, an infection describes the invasion of the pathogen into the human body, while inflammation is the response of the body towards the infection.

In most cases, the inflammatory response is sufficient to kill the pathogen and all is well once more. However, in some cases the response is unable to eliminate the pathogen at this early stage of infection. Another innate immune cell, the dendritic cell, will then start to play a vital role. This cell is continuously present in tissues and will phagocytose the pathogen. Unlike a macrophage and neutrophil, a dendritic cell's prime function is not to just phagocytose as many pathogens as possible. It will instead engulf only some pathogens, kill those, process them and then start displaying parts of the pathogen on its surface. This is called "presenting antigen" and is required to involve certain other immune cells. While the dendritic cell does this, it moves away from the site of injury towards the closest draining lymph node, which serves as a meeting point with other immune cells.[4, 5]

Lymph nodes are part of an intricate lymphatic system. In the skin, the lymphatic system works as follows. Fluid is constantly pushed out from capillaries into tissue at low volumes to supply tissue with nutrients and oxygen. Most of the fluid is reabsorbed by the blood vessels through osmotic pressure, but not all. The remaining fluid is sort of trapped and needs to be transported away, lest we would all swell up like balloons after a while. The fluid, now called lymph, flows into small vessels, called lymphatics. The lymphatics drain the lymph towards draining lymph nodes. In these lymph nodes, immune cells are present that "taste" the lymph for the presence of pathogens. Ultimately, the lymph will leave the lymph node via another draining lymphatic and flow towards the next lymph node. The lymph will pass several lymph nodes to ultimately drain into a major vein and thus back into the blood circulation, completing the circle.[4, 5]

Lymph nodes are not the only lymphoid organs in the human body. A distinction can be made between central or primary and peripheral or secondary lymphoid organs. In the central lymphoid organs, (adaptive) immune cells are produced, while in the peripheral lymphoid organs, (adaptive) immune cells start their activation process. Examples of central lymphoid organs are the thymus and bone marrow (BM). Examples of peripheral lymphoid organs/tissue are the lymph nodes, the tonsil, the spleen and Peyer's patches in the gut.[4, 5]

For our example of the pathogen, the dendritic cell is transported together with the lymph to a nearby draining lymph node. In the lymph node, the dendritic cell can interact with cells from the adaptive immune system via the displayed antigen. If an antigen is recognized, communication between the two arms is initiated (innate and adaptive). The adaptive immune system can now be activated and help destroy remaining pathogens that the innate immune system was unable to eliminate.

Before I move towards explaining adaptive immunity, there is one more aspect of the innate system that needs to be mentioned. Some pathogens have devised a method to avoid detection and being killing by the innate immune system. In the example so far, we have assumed that the pathogen stays outside of human cells, e.g. extracellular. However, viruses and some bacteria will invade a cell, hence they are called intracellular pathogens. This subterfuge will help hide them from the parts of the immune system that have been mentioned before.

Luckily, dendritic cells are not alone in their ability to present antigens at their surface. Almost all cells in the human body continuously present antigens. If there is no pathogen, antigens are presented from degraded proteins from within the cell, this is called presenting self-antigen. Thus, under normal conditions, cells only present self-antigens. However, if a cell is taken over by a virus or an intracellular bacterium, antigens from the pathogen will be presented on the surface. An infected cell is then visible and can be detected by the immune system.[4, 5]

To avoid this, some intracellular pathogens have evolved and developed an additional escape mechanism. Once these pathogens enter the cell, they prevent the infected cell from displaying pathogen-derived antigens on the surface by preventing the production of certain receptors.[10] Natural selection however, came up with a smart response to counteract this escape mechanism. A natural killer (NK) cell attacks and kills cells that lack (or display very low amounts of) the antigen presenting receptors on the surface. This is called the "missing-self hypothesis".[11, 12] An NK cell regulates its response with a combination of inhibitory and activating receptors.[13, 14] To conclude, the pathogen now faces a dilemma: downregulate the antigen presenting receptors of the infected cell and risk being killed by NK cells, or leave the receptors alone and risk being killed by cells from the adaptive immune system.

The system of antigen presenting receptors is called the human leukocyte antigen (HLA) system, or the major histocompatibility complex (MHC). The latter is the more universal way of addressing this system as it can be used for all animals, while the HLA system is specific for humans. In short, the HLA system is the collection of molecules that cells use to present antigen to the rest of the body. The antigens that can be displayed consist of small peptides which have been cleaved from larger proteins inside the cell. These large proteins can, as mentioned, vary from self-proteins, viral proteins or phagocytosed pathogen proteins.[4, 5]

There are two HLA classes. HLA class I binds and presents antigen that was produced within a cell, therefore, presenting the status of the inside of a cell. Almost all cells have HLA class I molecules on their surface. HLA class II binds antigen that was sampled from outside of the cell, demonstrating how the environment of the particular cell looks like. Only a certain number of specialized cells, called antigen presenting cells (APCs), have HLA class II molecules on their surface, dendritic cells being one of the important ones.[4, 5]

There are thousands of different HLA alleles (>9.000 class I and >2.500 class II alleles) currently known.[15] Each allele codes for a specific HLA receptor. The binding sites of the HLA receptor determine the peptide it can bind and thus present to its environment. Different HLA receptors can bind and present different peptides from the same pathogen. Each individual has only a select number of HLA alleles, as such a large variety of combinations of alleles is possible across the human species. Some HLA alleles are however more common within certain populations than other HLA alleles. Since some HLA receptors are better at binding antigens from certain pathogens, these receptors are more prevalent in areas where these pathogens are endemic.[16] An evolutionary selection of HLA alleles based on endemic pathogens in a region can be observed. Therefore, while the chance of two unrelated individuals having the exact same set of HLA alleles is small, it is not impossible. For siblings, the chance of having the same HLA alleles is much higher; 1 in 4, as we receive half of our HLA haplotypes from each parent.[4, 5]

At this point I feel that I must point out that there are several more aspects of the innate immunity that are important for its function that I have not mentioned yet. For instance, the innate system actually relies quite heavily on pattern recognition receptors such as Toll-like receptors and NOD-like receptors to identify pathogens. Another example is the complement system, which is also a part of the innate immune system. The complement system functions as a cascade of small soluble proteins which ultimately can kill pathogens by punching holes in their membranes or by making pathogens appear more recognizable as such for other immune cells.[4, 5] I will not discuss these aspects of the innate immunity in detail as they fall outside the scope of this thesis.

In short, the innate system plays a vital role in our survival. Individuals without an innate immune system or a severely dysfunctional innate system do not survive for long. The main reason for this is that the innate system is continuously patrolling and can act within minutes, while the adaptive immune system is slow in response and needs several days to mount an effective response. The major disadvantage of the innate immune system is its inability to adapt and change its response to better fit the pathogen in question. The adaptive immune system adapts (hence the name) its response to the pathogen and becomes more specific and effective as time goes on.

1.1.2 Adaptive Immunity

As mentioned before, during an infection, dendritic cells from the innate immune system will phagocytose and process pathogens. They then migrate to the closest lymph node and start to present pathogen derived antigens on their HLA molecules. In the lymph node, the dendritic cell will encounter T and B lymphocytes, which are present in separate areas of the lymph node. These two cell types, and their many subtypes, form the adaptive immune system.[4, 5]

1.1.2.1 T Lymphocytes

T lymphocytes, or T cells, all have the T cell receptor complex (TCR) on their surface. With these TCRs they can interact with the HLA receptors on other cells. Each T cell displays multiple copies of the same TCR on its cell surface. Each TCR can recognize only one specific antigen, hence, each T cell is specific for only a single antigen. We have several millions of different specific T cells in our bodies. Only a few will ever encounter their specific antigen, the vast majority will never encounter "their" antigen in their lifetime.[4, 5]

The TCR (Figure 3) consists of a collection of protein chains with both extracellular and intracellular domains. The binding chains of the TCR are extracellular and are anchored into the cell membrane. They consist of an α and β chain which together interact with the antigen presented by the HLA receptor. Most specifically, the outmost part of the extracellular domains interacts with the HLA receptor as this is the variable region. The lower part forms the constant region and anchors the chains into the cell membrane.[4, 5]

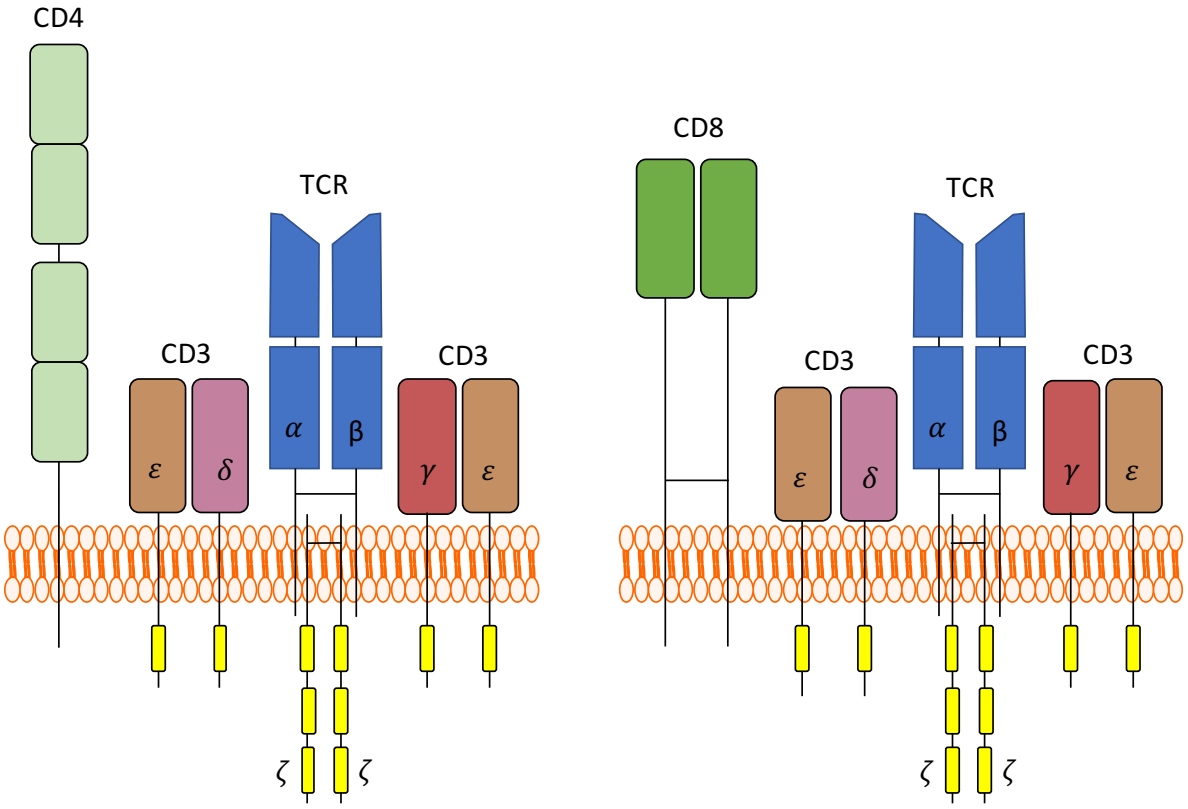

Figure 3. Schematic of the TCR complex. In the left panel, a CD4+ T cell is depicted and on the right a CD8+ T cell.

The TCR complex also contains the cluster of differentiation (CD)3 complex. The CD3 chains are the signalling domains that start a cascade of activation internally if a TCR binds to an HLA receptor and recognizes the antigen presented by it. CD3 is expressed on all T cells at a high number and is therefore a good marker for analysis.

Each T cell has a unique α and β chain and can thus recognize unique antigens. However, the TCR first needs to recognize and bind to the HLA receptor. For this it uses two co-receptors (CD4 or CD8) associated to the TCR. A mature T cell, generally, can only express CD4 or CD8, not both, and are classified to be either CD4+ or CD8+. CD4+ and CD8+ T cells have very distinct functions which will be discussed in more detail later on.

One of the differences is the fact that CD4+ and CD8+ T cells bind different classes of HLA molecules. CD4+ T cells recognize HLA class II and CD8+ T cells recognize HLA class I molecules.[4, 5]

T cells have to undergo a maturation and education process to ensure a huge variety in TCRs. This development is a complicated and lengthy process. In short, T cells start their development in the BM, together with B and NK cells. The T cell progenitors migrate towards the thymus where they rearrange their TCR genes and become formally educated. During this process, unwanted T cells, for example autoreactive T cells, are removed from the T cell repertoire. After this, the cells are primed for function. The T cells migrate from the thymus towards the peripheral lymphoid organs, such as the lymph nodes, in search of their antigen. Upon encountering their antigen, the T cells are activated, proliferate and migrate towards the site of infection where they will engage the pathogen. Each of these phases in T cell development will be discussed in some detail below.

T cells are formed from a common NK/T/B cell precursor in the BM, called the common lymphoid progenitor (CLP). Some of these progenitors leave the BM and migrate towards the thymus. These progenitors do not yet express CD3, as they have not yet formed a TCR. They also do not yet express CD4 or CD8, and are often called double-negative thymocytes. Upon arriving in the thymus, the double negative thymocytes differentiate and rearrange their TCR genes. During this rearrangement, or somatic recombination, ultimately the TCR complex is formed. The cells start by simultaneously rearranging the γ,δ and β TCR genes. During this rearrangement random segments of V, D and J gene segments for the δ and β chains, and V and J gene segments for the γ chain of the TCR are combined. This ensures that a large variety of potential TCRs are created. If a TCRγδ is formed first, the T cell will become a γδ T cell, leave the thymus and move into the periphery. However, the vast majority of cells will first form a stable pre-TCR with only the β chain, thus committing the T cell to become an αβ T cell. As most T cells will become αβ T cells, I will continue explaining their development. The thymocytes with a stable pre-TCR will now undergo extensive proliferation to ensure that many thymocytes with the same pre-TCR exist. At the end of this proliferation, the cells will also start to display both CD4 and CD8 on the surface in conjunction with the pre-TCR. The thymocytes are now referred to as double-positive thymocytes. The α TCR gene is then rearranged and this continues until a stable TCRαβ is formed to replace the pre-TCR containing only the β chain. Since the α chain rearrangement is random and since there were many thymocytes with the same pre-TCR after the proliferation, an enormous variety of αβ T cells are created. The thymocytes are now ready to be educated.[4, 5, 17, 18]

The thymus does not only contain thymocytes, it is also populated with stromal cells that display almost any imaginable self-antigen on their HLA (class I and II) molecules. These stromal cells are essential for T cell education. The thymocytes are educated via a process called positive and negative selection. During positive selection, the thymocytes will move around the stromal cells and try to bind to the HLA molecules via CD4 or CD8. The thymocytes that can recognize the HLA molecules will bind and receive a survival signal. Those that are unable to bind HLA will not receive a survival signal and instead go into programmed cell death, a process known as apoptosis. During this selection only T cells that can recognize the body's HLA molecules remain. This is crucial, as T cells need to be able to recognize the HLA molecules in the periphery, or they will never be activated. During this process, the thymocyte will also commit to be either a CD4+ or CD8+ T cell. If the thymocyte binds HLA class I first with the CD8 co-receptor, the T cell will become a CD8+ T cell and vice versa for HLA class II and CD4. The unstimulated co-receptor will be downregulated.[4, 5, 17-19]

The single positive thymocytes that have survived move onwards to the negative selection process. In this phase, they again face the stromal cells displaying self-antigen on HLA molecules. This time, they are tested for their potential to recognize and bind self-antigen displayed on the HLA molecule. The thymocytes that bind too strongly will receive an apoptosis signal as these are potentially self-reactive T cells. Consequentially, those that do not bind, or very weakly bind, do not receive an apoptosis signal and will survive. At the end of the negative selection we are left with single positive thymocytes that can recognize self-HLA but do not recognize self-antigen.[4, 5, 19] The thymocytes are now released from the thymus and move into circulation. They are now so-called naïve T cells.

Thymal education is an ongoing process throughout life although it is severely diminished as we grow older. The thymus starts to degenerate during adolescence and thymic tissue is gradually replaced by fat, diminishing thymic function as we age.[20]

The naïve T cells migrate between the blood and lymph nodes in search of their antigen. After encountering and interacting with a dendritic cell presenting their antigen in a lymph node, they will become activated. For a naïve T cell to become activated for the first time, just recognizing the antigen displayed on HLA is not enough. The naïve T cell will also need a co-stimulatory signal, most often in the form of CD28 on the T cell, binding to a co-stimulatory molecule (e.g. B7) on the dendritic cell.[21, 22] Therefore, the dendritic cell needs to not only present the pathogen antigen, it also needs to present co-stimulatory signals to convince the naïve T cell to activate. This is a protective function, to ensure that naïve T cells will not attack tissue unless they receive a strong co-stimulatory signal indicating something is wrong.

Binding of the antigen-HLA complex to the TCR and costimulatory complex sets in motion an activation cascade. A series of intracellular domains are phosphorylated, recruiting proteins like zeta-chain-associated protein kinase 70 (ZAP-70) and lymphocyte-specific protein tyrosine kinase (LCK), which continues the downstream signal towards the cell nucleus (Figure 4). In short, the TCR binds and recognizes the antigen-HLA complex. The co-receptor (CD4 or CD8) then binds to the HLA molecule, leading to a conformational change. LCK is recruited to the intracellular part of the co-receptor and is activated. LCK phosphorylates certain areas (immune-receptor tyrosine-based activation motifs (ITAMs)) on the intracellular parts of the TCR complex. The changes to these areas now allow ZAP-70 to bind, resulting in its phosphorylation and activation by LCK. The phosphorylated ZAP-70 then goes on to phosphorylate other signalling proteins, propagating the signalling cascade. In parallel, CD28 binds to another receptor (B7) on the APC, resulting in phosphorylation of the intracellular part of CD28. This then results in a cascade of recruitment and phosphorylation of several other proteins, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) being one of the first. Ultimately, transcription factors and cytokines are produced resulting in cellular activation.[4, 5, 23] For the sake of simplicity this introduction will not delve deeper into the mechanisms of TCR activation.

After a naïve T cell has been activated it clonally expands and differentiates from a naïve T cell into a central memory and/or an effector memory T cell. The effector memory T cells fight the infection, while the central memory T cells function as the immunological memory. These central memory T cells can clonally expand quickly should the pathogen invade again and will not need a costimulatory signal to do so the second time. This is part of the reason why we are usually ill for a longer period the first time we encounter a pathogen and a shorter period the second and third time we encounter the same pathogen. Additionally, this memory development is the rationale behind vaccinations. By

immunizing an individual with a dead or weakened pathogen, the adaptive immune system can develop memory against the pathogen.

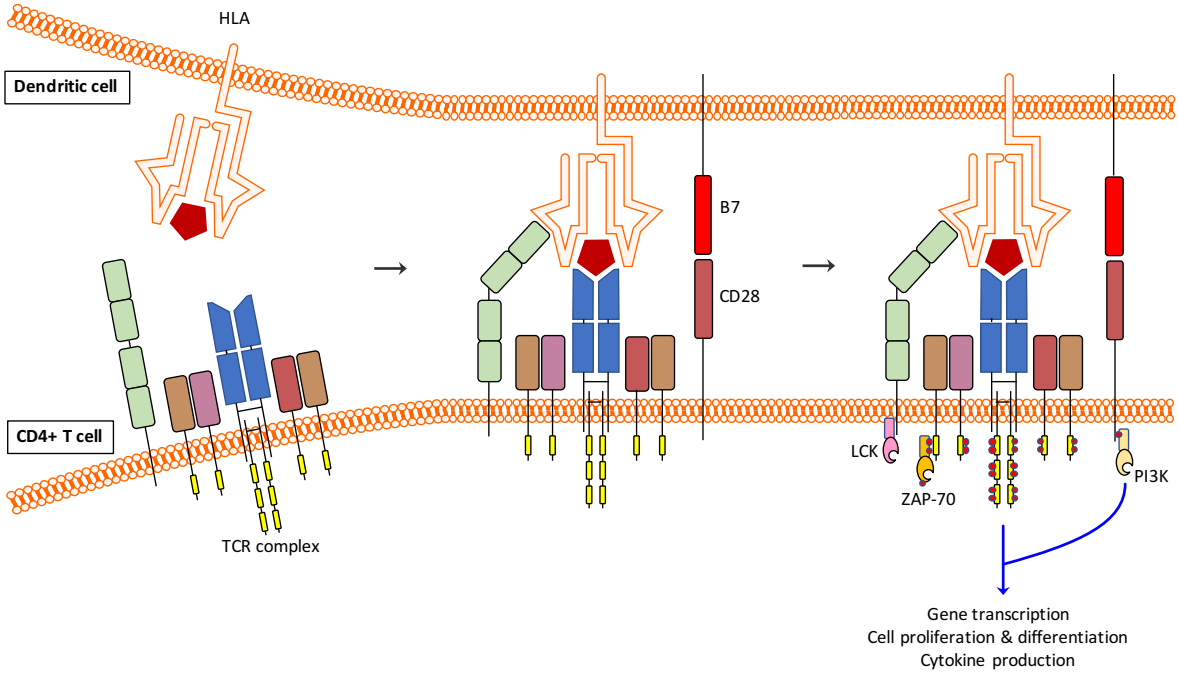

Figure 4. A representation of the activation of a CD4+ T cell upon encountering its antigen as presented by an HLA class II molecule on a dendritic cell.

There are two commonly used models for T cell memory differentiation. In the first model, the off-on-off model, naïve T cells differentiate into effector memory T cells. After an infection has been removed, most effector memory T cells die but some turn into central memory T cells. If an infection reoccurs, the memory T cells can then turn back into effector T cells. The second model, the developmental model, states that naïve T cells differentiate into central memory T cells first. Some of these central memory T cells will then differentiate into effector memory T cells that will fight the infection. The effector memory T cells will then go into apoptosis after the infection is cleared. Evidence for both models can be found and further research elucidating the exact mechanism is still needed, though most evidence does point toward the developmental model. For research purposes, expression of the cellular markers chemokine receptor (CCR)7 and CD45RO/CD45RA is most often used to classify the differentiated cell types.[24]

After a sufficient number of effector memory T cells have been formed, the T cells will either interact with B cells or leave the lymph node in search for the pathogen. Interaction of T and B cells will be explained in more detail later on. The effector T cells that leave the lymph node are homed through chemotaxis by chemokines that are released at the site of infection. When they encounter the pathogen they no longer need a co-stimulatory signal to act.[4, 5]

T cells come in many, figuratively speaking, shapes and sizes. New subtypes are continuously being discovered. We will focus on some of the most abundant subtypes and the subtypes most relevant to the contents of this thesis. Conventional T cells can foremost be divided into CD4+ and CD8+ T cells. These two T cell subtypes are also termed T helper (Th) cells (CD4+) and cytotoxic T cells (CD8+).

CD4+ T cells are called T helper cells as they mainly facilitate the communication and activation of immune cells (Figure 5). Th cells come in varying subtypes; Th1, Th2, Th17, follicular helper T cells (Tfh) and regulatory T cells (Tregs) being the most commonly described. This classification is mainly based on the cytokines they excrete and the effect those cytokines have on other immune cells.

Th1 cells are focused on intracellular pathogens, bacteria and viruses. They mostly produce interferon (IFN)γ which stimulates macrophages to more effectively phagocytose and destroy intracellular pathogens. Th2 cells usually excrete interleukin (IL)-4, IL-5 and IL-13. These cytokines influence the function of other types of cells which are mostly associated to the defence against extracellular parasites. For instance, activation of mast cells and promotion of B cells to isotype switch to IgE. In short, Th1 cells are focused on cytotoxic or cellular immunity, while Th2 cells promote humoral immunity, via B cells. Th17 cells produce IL-17, which stimulates neutrophils and helps the immune system to fight extracellular bacteria and fungi. Tfh cells are important in the communication with B cells. These cells form germinal centres with B cells in the lymph nodes where they can influence B cell maturation. This process will be described in more detail later on. Lastly, Tregs have an immune regulatory function. They dampen the immune response in the periphery to ensure that our own immune response does not end up killing us. They are an integral part of a critical negative feedback loop.[4, 5, 25, 26]

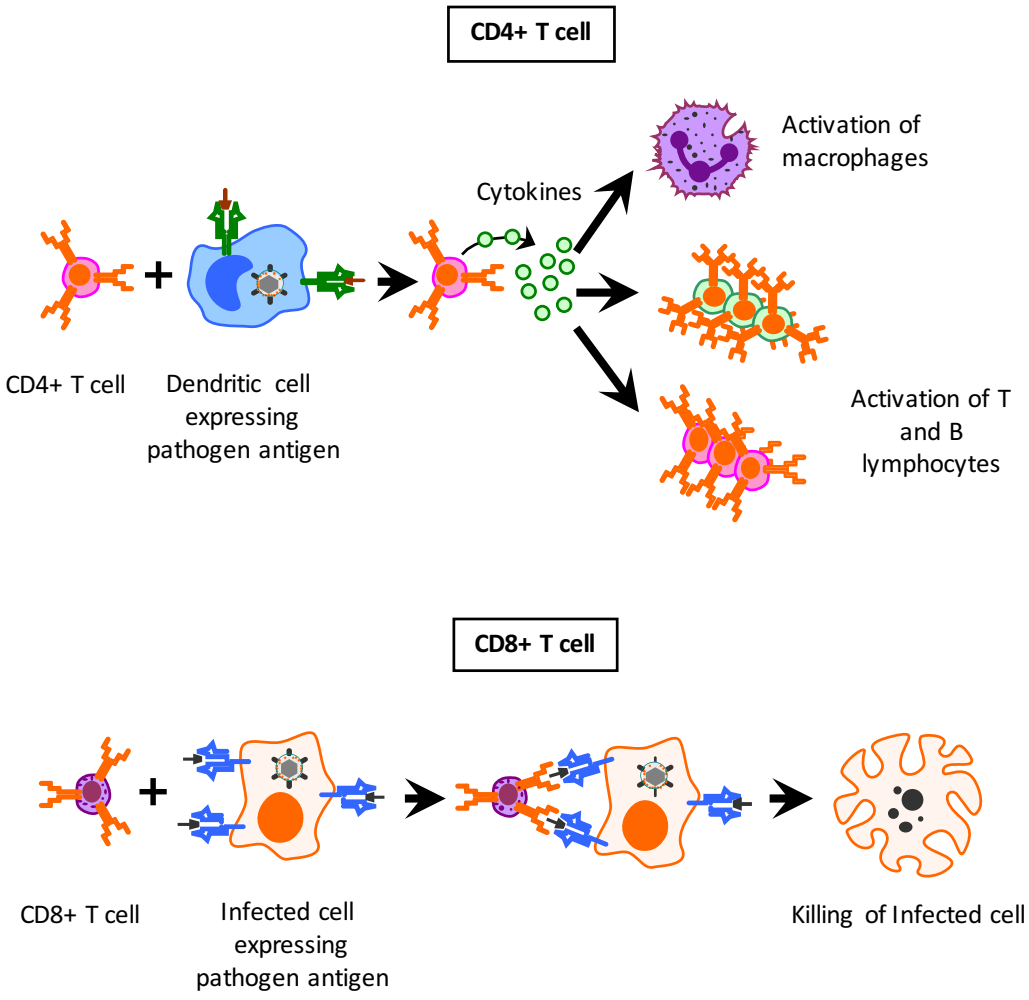

Figure 5. Schematic representation of some functions of CD4+ and CD8+ T cells.

In contrast to CD4+ T cells, CD8+ T cells do not come in as many subtypes (as far as we know). These cells are similar to NK cells, as in they respond to intracellular threats. Upon activation, they will migrate around the body in search for cells that have been invaded by the pathogen. If they recognize an infected cell, they will destroy it and the pathogen inside of it (Figure 5). They kill by releasing perforins and granzymes which punch holes in the cell membrane and induce apoptosis after entering the cell, respectively. While CD8+ T cells are invaluable in destroying intracellular pathogens, they are potentially also one of the most dangerous immune cell subsets to be autoreactive.[4, 5]

There are also several non-conventional T cells, though these are present at much lower frequencies than conventional CD4+ and CD8+ T cells. One of these types of T cells are the so-called mucosal-associated-invariant T (MAIT) cells. These MAIT cells are a relatively new discovery and can be characterized by their expression of CD161 as well as a specific TCR receptor, TCRVα7.2-Jα33.[27-30] They are found at low frequencies in the blood, around 5% of total lymphocytes, but are present in much higher frequencies in mucosal areas of the body, hence their name. These cells do not respond to traditional peptides like conventional T cells. Instead, they recognize and are activated by riboflavin metabolites, most commonly formed by biosynthetic pathways in bacteria and yeasts. It is probably for this reason that MAITs can be found more in mucosal areas, where bacteria are more abundant.[31, 32]

Another subtype of cells are the invariant NKT cells (iNKT). These iNKT cells constitute less than 1% of the blood T cells. They have most of the same receptors and markers as NK cells, but also have a TCR, making them T cells and not NK cells. Unlike conventional T cells, they do not recognize HLA, but are instead CD1d-restricted (an MHC-like molecule). Because of this, these cells recognize lipids instead of peptides.[31, 33]

Lastly, all T cells discussed so far are T cells with a traditional α and β chain TCR. There is a subset of T cells that instead have a γ and δ chain, as briefly alluded to in the section on T cell development. These $\gamma\delta$ T cells do not undergo education in the thymus, but instead mature in the periphery. The exact mechanisms of their education are not entirely understood yet. Interestingly, $\gamma\delta$ T cells do not seem to be restricted to HLA for activation, such as $\alpha\beta$ T cells are. While it is not entirely clear how $\gamma\delta$ T cells are activated, they seem to recognize lipids instead of proteins, similar to iNKT cells.[31, 34]

To conclude, T cells are essential for a successful immune response. They have an extremely varied response and recognition repertoire. They are vital, but they are not alone. They receive and give a lot of help to B lymphocytes, the other important cell type of the adaptive arm.

1.1.2.2 B Lymphocytes

B lymphocytes, or B cells, have a B cell receptor (BCR) on their surface (Figure 6). The BCR is essentially a membrane bound immunoglobulin (Ig). B cells are identified by their expression of CD19, a marker that, unlike the incorporation of CD3 in the TCR on T cells, is not incorporated into the BCR on B cells. Instead, it resembles the function of CD4 and CD8 in T cells, since it strengthens the B cell activation cascade when engaged. CD19 forms, together with CD81 and CD21, the B cell co-receptor complex (Figure 6).[4, 5]

A B cell's main function is to produce and secrete antibodies, which are essentially soluble Igs. Antibodies recognize pathogens directly and not via HLA(-like) molecules like T cells

do. Antibodies can be seen as a combination of two heavy chains and two light chains (Figure 6). During a process called somatic recombination, B cells rearrange their heavy and light chain gene segments and start to express a large variety of potential heavy and light chains, somewhat similar to TCR gene rearrangement. Similar to T cells, the rearrangement of the heavy and light chains is random. This creates a vast variety of B cells with different heavy and light chains, but a single B cell will only produce a single combination of the chains, producing a single specific antibody.[4, 5]

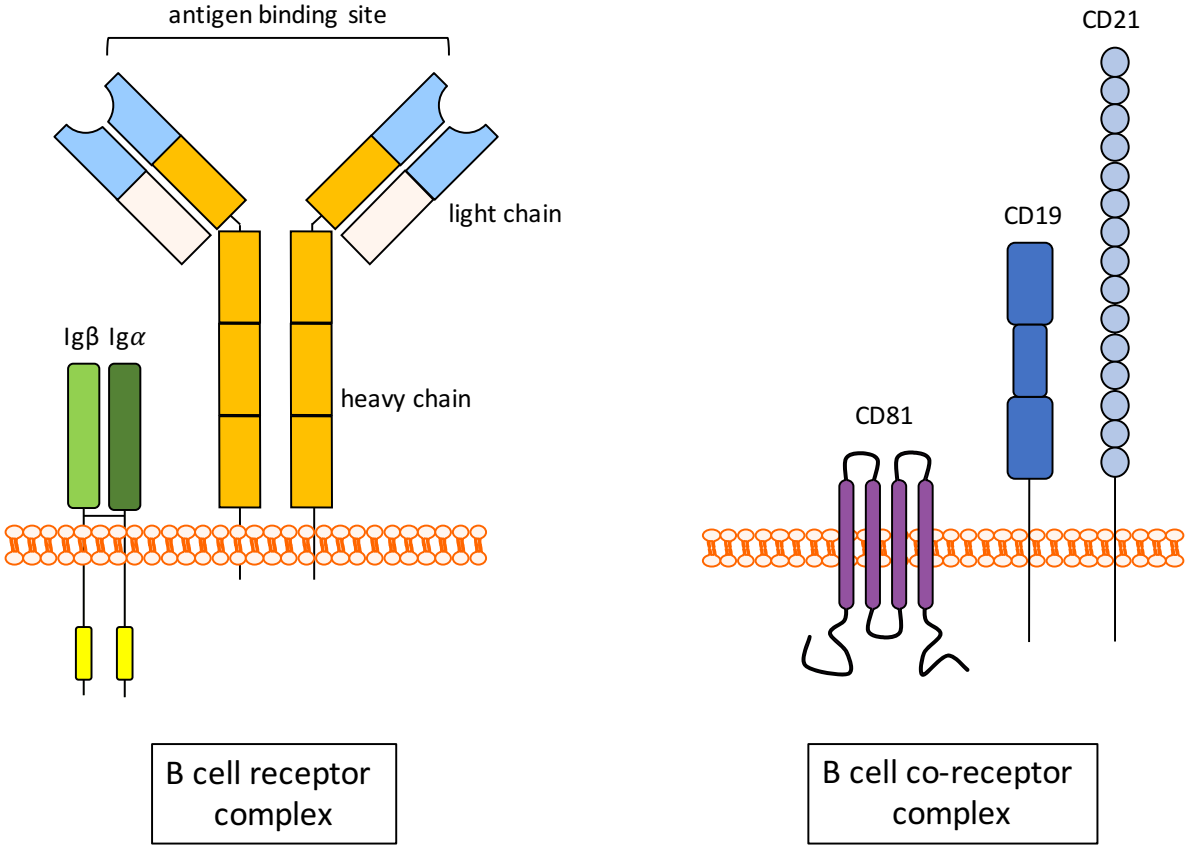

Figure 6. Schematic of the B cell receptor complex (BCR) and the B cell co-receptor complex. The BCR, consists of an immunoglobulin which can recognize and bind a specific pathogen antigen. The associated Igα and Igβ will start the signalling cascade to activate the B cell after the immunoglobulin binds its antigen. The B cell co-receptor complex consists of a CD81, CD19 and CD21 receptor. After CD21 binds to complement C3dg coated on the pathogen surface, the B cell co-receptor complex clusters with the BCR. This phosphorylates CD19 and initiates downstream signalling to enhance cell activation. The role of CD81 is as of yet unknown.

Antibodies can also be split into a variable and a constant region. The variable region recognizes the pathogen and is formed by both the light chain and the top part of the heavy chain. Since a large number of combinations of heavy and light chains are possible, the variable region varies dramatically between B cells. B cells can thus recognize a large number of pathogens.[4, 5]

The constant region determines the antibody class or isotype, and is formed by the lower part of the heavy chain. There are 5 Ig isotypes; IgA, IgD, IgE, IgG (which has 4 subclasses of its own: IgG1, IgG2, IgG3 and IgG4), and IgM. The Ig isotype determines its function and efficacy. For instance, IgM, IgG1 and IgG3 can start the classical pathway and IgA the alternative pathway of complement activation.[35] IgE is involved in defence against multicellular parasites but also plays a role in allergy.[36] While B cells may always produce the same antibody in terms of the pathogen pattern they recognize, during development and

activation they can and will switch isotypes. This isotype switching ensures a more efficient response adapted to the specific pathogen.[4, 5]

B cell development and activation are quite different compared to T cell development. One main difference is the site of development. B cells start their development in the BM, where they will stay until they become mature naïve B cells and are released into the periphery. They continue their education and development in lymphoid tissues, influenced by CD4+ T cells and dendritic cells. Since B cell development is not directly dependent on the thymus, new mature naïve B cells are continuously formed throughout life.

B cells have the same progenitor as T and NK cells, however, they mature in the BM. The common lymphoid progenitor either leaves the BM towards the thymus to become a T cell, or it interacts with the stromal cells in the BM, directing the cell to go down the B cell developmental pathway. During B cell development, the B cell migrates through the BM and progresses through the following steps; early pro-B cell, late pro-B cell, large pre-B cell, small pre-B cell, immature B cell and finally a mature (naïve) B cell. During these stages, random heavy and light chain rearrangement takes place. If the B cell is incapable of forming a stable heavy and light chain combination, the B cell will undergo apoptosis. During development, B cells are in constant contact with the stromal cells until they reach the stage of immature B cell. Similar to T cells, B cells need to undergo negative selection. Potential autoreactive B cells must be eliminated as they otherwise can cause harm to our bodies. This process is called central tolerance, as the B cells are tested for autoreactivity in the BM, a central lymphoid organ. Some autoreactive B cells may escape this process and move towards peripheral lymphoid organs. Luckily, B cells can also be removed here through the process of peripheral tolerance. Ultimately, the aim is to have cells capable of recognizing pathogen antigen and not self-antigen. If an immature B cell survives the central tolerance process, the cell continues to mature and will leave the BM. The mature naïve B cell now displays both IgM and IgD on its surface. The B cell will need to be activated before it can produce other isotypes.[37-40]

After leaving the BM, the mature naïve B cell moves into the peripheral lymphoid organs where it can interact with dendritic cells. After a mature naïve B cell has encountered its antigen on a dendritic cell, the B cell internalizes the antigen-Ig complex and starts to display the antigen on HLA class II receptors.[4, 5] The B cell can now interact with CD4+ T cells. It needs to do so in order to receive additional signals required for activation. If it does not interact with a CD4+ T cell within 24 hours it will go into apoptosis. The B cell and the CD4+ T cell recognize the same antigen and begin to interact. They migrate together to a specific location in the lymph node and start to clonally expand forming so-called germinal centres.[41] Depending on the interaction with CD4+ T cells (production of certain cytokines), B cells will switch the constant region of their Igs. This leads to a change in isotype class, a process called isotype switching. Moreover, unlike T cells, B cells can also diversify the variable regions of their Igs. This is done through somatic hypermutation and gene conversion. They both alter the variable region of an Ig leading to an altered recognition of the antigen. Somatic hypermutation introduces point mutations in the heavy and light chain variable regions, while gene conversion will replace entire segments of the variable region with segments from variable regions of certain pseudogenes. The end result will affect the B cell antibody affinity for the antigen, with some binding better and others worse. Due to competition for available antigen, B cells with antibodies that bind better will outcompete the other B cells. Thus, the B cells with optimized antibodies survive.[42, 43] This entire process of T-B cell interaction usually starts days after primary infection and lasts for several more days. Therefore, it takes between 1-2 weeks after initial primary infection before a robust B cell response is in place.[4, 5]

After the B cells have been activated, expanded and optimized, they either become memory B cells or plasma cells. Plasma cells migrate back towards the BM and essentially become antibody-secreting factories. These cells continuously secrete antibodies and function as a line of defence for a recurring pathogen invasion until they ultimately die.[44] The central memory B cells migrate from lymph node to lymph node in search of a new invasion of the same pathogen. They will be able to expand and react to a new infection more quickly and efficiently the second time as they no longer need to undergo isotype switching or increase their specificity.[4, 5]

B cells fight pathogens by coating the pathogen of interest with secreted antibodies. B cells secrete antibodies, which then circulate in the blood and lymph in search of a pathogen they can bind. If a pathogen is found, the antibodies bind to its specific target on the surface and start to coat it. This coating has multiple effects (Figure 7). First, coating the pathogen with antibody may make it impossible for the pathogen to further infect other cells or for a toxin to be toxic. This is called neutralisation. Secondly, the coated antibodies also make the pathogen more easily recognizable for macrophages and neutrophils. When coated, the pathogen is more easily phagocytosed and killed by these cells through the process of opsonisation. Lastly, the antibodies coated to the surface of the pathogen may attract certain proteins of the complement system. Complement proteins are an additional pathway to phagocytosis as an accumulation of complement proteins on a pathogen is also attractive for phagocytes. Moreover, the complement system can ultimately create holes in the surface of the pathogen, thus killing it. Larger parasites which cannot be phagocytosed can be killed in this manner. B cells and their antibodies are thus vital for the elimination of extracellular pathogens.[45]

In conclusion, during activation, B cells go through a process where they recognize their pathogen antigen better and better. This is a major difference compared to T cells. After a T cell has finished education in the thymus, the T cell has reached its maximum potential to recognize antigen. A mature B cell can improve its antigen recognition after encountering its antigen.

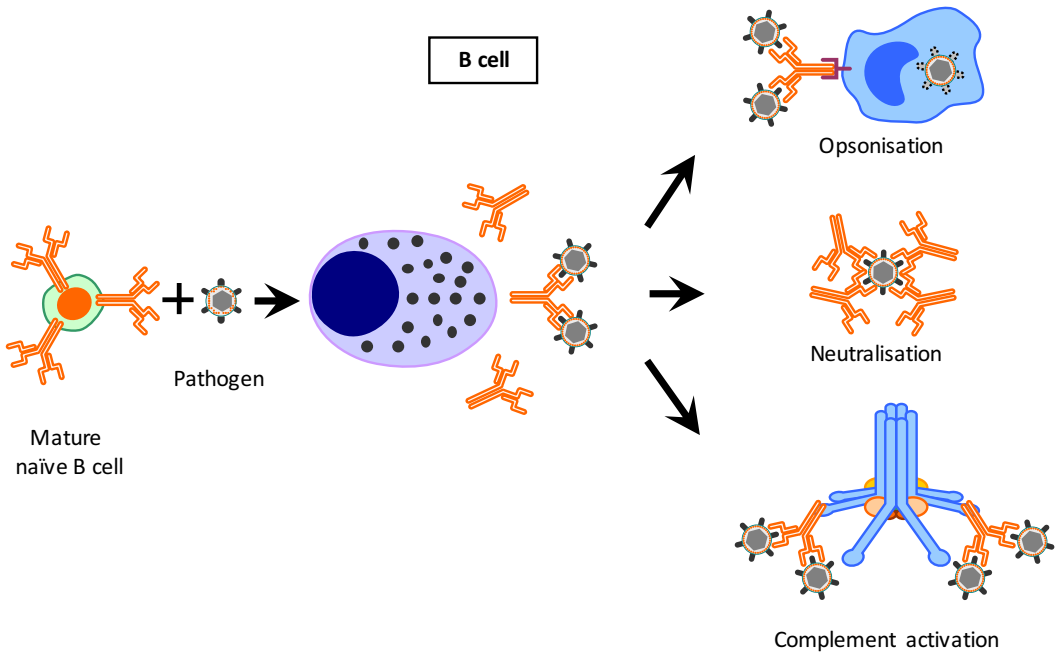

Figure 7. Schematic representation of the function of B cells.

1.1.3 When the System is Broken

For our continued survival, we all need a well-functioning immune system. For the immune system to work correctly, it needs to be able to discriminate between "self" and "non-self" as well as "normal" and "non-normal". Unfortunately, it occasionally happens that the immune system struggles with this discrimination.

An example of this can be seen in the form of autoimmunity disorders, such as rheumatoid arthritis. In autoimmune disorders, a patient's immune system mistakes healthy normal cells for foreign or non-normal cells and attacks, resulting in the destruction of perfectly healthy tissue.[46] The reverse is also possible, the immune system fails to recognize non-normal cells or is incapable of destroying the non-normal cells, leading to a proliferation of cells that should not be there. This is what happens in cancers (in simple, broad terms).[47] Some people are even born with a deficiency in their immune systems; e.g., primary immunodeficiency disorders (PIDs). The immune system in these patients is severely impaired.[48]

There are also other things that can influence the proper functioning of the immune system. For instance in the case of human immunodeficiency virus (HIV). HIV does not result in the death of patients directly, instead, people die from common pathogens. Normally, these pathogens would be no problem for the immune system to handle. However, in the case of HIV, the immune system is compromised as the patients' CD4+ T cells are infected and destroyed by the virus, resulting in very low frequencies of this vital cell type. The patient's adaptive immune system is therefore reduced in function and is heavily compromised in its ability to eliminate common pathogens.[49]

So far, these examples have all been natural causes for immune impairments. It is also possible to impair an immune system in a more man-made way; irradiation. After the nuclear bombs on Nagasaki and Hiroshima at the end of the Second World War, survivors were exposed to large doses of radiation caused by the fall-out of the bombs. As a result, their immune systems were almost completely destroyed. With the cold war starting and people fearing a global nuclear war, scientists started exploring ways of transplanting immune systems from a donor to a patient whenever a patient was exposed to very high levels of radiation.[50] Such an immune system transplantation is more accurately termed a hematopoietic stem cell transplantation (HSCT). The entire hematopoietic system is transplanted, which includes all blood cells and not just immune cells (Figure 1).

Coincidentally, nuclear bombs were not the only cause for irradiation. Clinicians had also been experimenting treating malignancies, such as cancers, with irradiation. All of this research led to the start of the field of HSCT and transplantation immunology.

1.1.4 Transplantation Immunology

Nowadays, transplanting organs or hematopoietic stem cells from a donor to a patient is done on a regular basis in many hospitals across the world. Most countries even advertise voluntary donor programs. However, transplantations were at one point experimental and dangerous. Fine-tuning transplantation procedures has taken time. Since the start of this field in the 1960s, there has been a significant research effort to better understand transplantation immunology. For instance, we now know that we cannot indiscriminately place any donor's organ into any patient. We need to "match" donor and patient for certain variables, how stringent depends on the organ or tissue transplanted. This necessity of matching comes down to immunology.

The immunology behind transplantation is complicated. The most important factor in transplantation immunology is the fact that immune systems are trained to recognize "self" from "non-self". By definition, a donor's organ is "non-self" and will be attacked by the patient's immune system if left to its own devices. Similarly, in a HSCT, the entire patient body is seen as "non-self" by the transplanted donor immune system.

The consequence is that patients who receive a donor solid organ (liver, kidney, heart, etc.), will usually have to take lifelong immunosuppressive drugs to keep their immune systems from attacking and rejecting the new "non-self" organ.[51] Physicians have to balance suppressing the patient immune system to not reject the transplanted solid organ while allowing sufficient leeway for the immune system to function so patients do not get terminally ill from common pathogens. For HSCTs this works slightly different, yet similar in many ways, and will be explained in detail in the next sections.

1.2 HEMATOPOIETIC STEM CELL TRANSPLANTATION

1.2.1 Rationale & History

As mentioned earlier, scientists first started to consider HSCTs after the two nuclear bombs were dropped over Japan at the end of the Second World War. Clinicians were trying to treat patients who were severely immunocompromised, anaemic and thrombocytopenic due to the high radiation. At the same time, they realized that the ability to rescue these patients could be an extremely valuable treatment option for patients with malignancies like cancer. Cancer treatment at the time was hampered by the fact that clinicians could only irradiate patients to a certain level lest their hematopoietic system became too heavily compromised resulting in patient death. This meant that treatments were often inefficient as cancers came back. If they could find a way to cure patients with almost no remaining hematopoietic system, they would be able to irradiate patients with cancers to a much higher degree.

The first HSCT experiments were performed on animals, mostly in mice and dogs, in the 1950s and early 1960s.[52, 53] In the late 1950s and 1960s, the first trials on humans were done.[50, 54] At that time the HLA system was not yet discovered. Unsurprisingly, patients who were transplanted in this early era of HSCT did not survive for long. Main causes of death were graft failure, graft-versus-host disease (GVHD), relapse and infections, all of which will be explained in detail later on.[54, 55] Due to the disappointing results, the field stagnated and not many HSCTs were performed on humans.

However, after the scientific community learned more about the immune system and specifically about the HLA system[56], HSCTs were performed increasingly often. Several landmark achievements (for instance the discovery of immunosuppressive drugs like cyclosporine) further improved survival rate and ultimately increased patient quality of life. HSCTs were becoming an established treatment option. Since the 1970s the field has thus been steadily growing, especially so during the last few decades. Improved HLA-typing methods, larger donor registries, more diverse conditioning regimen options, better immunosuppressive regimens and better supportive care have all contributed to that effect.[57] Currently, worldwide around 50.000 HSCTs are performed annually, curing a variety of disorders (www.who.int).[58]

1.2.2 Indications

The first disorders which were attempted to be cured by HSCT were mostly of a malignant nature, namely acute leukemias. However, HSCT was also quickly indicated for patients suffering from non-malignant disorders such as severe aplastic anaemia and severe combined immunodeficiency.[59, 60]

Currently, a large variety of both malignant and non-malignant disorders are treated with HSCTs. The vast majority of HSCTs worldwide are still done for malignant disorders, of which leukemias remain the most common.[61] The classification of leukaemia depends primarily on the developmental stage and lineage of haematopoiesis the leukemic cell is from. Acute myeloid leukaemia and chronic myeloid leukaemia both originate from the myeloid lineage. There are also leukemias that originate from the lymphoid lineage; acute lymphoid leukaemia and chronic lymphoid leukaemia (CLL).[62] CLL is also often referred to as B-CLL, as in this form of leukaemia the B cells are affected. These four leukemic types have several subtypes, mostly classified by genetic markers, but we will not go into depth about these variants. While these four leukemic types are the most common malignant disorders that can be cured by HSCT, there are several other malignancies for which patients are transplanted. These are for instance, myelodysplastic syndrome, lymphomas, multiple myeloma and several solid tumours.[61, 63, 64]

While malignant disorders are clearly lethal diseases if left untreated, non-malignant disorders are, despite the perhaps misleading name, also lethal in many cases, otherwise they would not warrant a HSCT, with its potential serious complications to be performed. Non-malignant disorders are generally divided into PIDs, non-malignant hematologic conditions and inborn metabolic disorders. These disorders can often only be cured with a HSCT. Though not exclusive to children, the vast majority of patients transplanted for non-malignant disorders are children or young adults. This is because most non-malignant disorders are present from birth. Some examples of non-malignant disorders that could be treated by HSCT are sickle cell anaemia, thalassemia, severe aplastic anaemia, Fanconi anaemia, Wiskott-Aldrich syndrome, and even severe forms of Crohn's disease.[61, 63-66]

1.2.3 Procedure

The decision whether to perform a HSCT is based on an individual patient assessment. Factors such as age, comorbidities, prior treatment, remission status and underlying disorder are examples of facts that must be taken into account. As HSCT is a high risk high reward endeavour, risk-benefit ratios must be considered for each patient.

While HSCT is a well-established technique performed in many countries, procedures are complicated and vary between centres. This section is an attempt at illustrating some of the aspects of HSCT procedure, though, due to space restrictions, not all aspects are touched upon. Aspects most vital for the understanding of HSCT in general and for the research performed in this thesis have been prioritized.

In short, the procedure for a HSCT is as follows. Hematopoietic stem cells are harvested from a healthy living donor. The patient undergoes a conditioning regimen and the donor hematopoietic stem cells are infused as a transfusion into the patient's blood stream. The patient is then monitored closely for early treatment and transplant-related complications and if all goes well, the patient should be able to go home after a few weeks. However, the continuous follow-up at a specialized open-clinic is usually life-long, even if visits may be less frequent over time if the post-HSCT period continues to be uneventful and free from

severe complications. For a simple graphical representation of the HSCT process, please see Figure 8. Of course, much more goes into a successful HSCT. Some of the details of each of these aspects will be discussed in detail in the next sections.

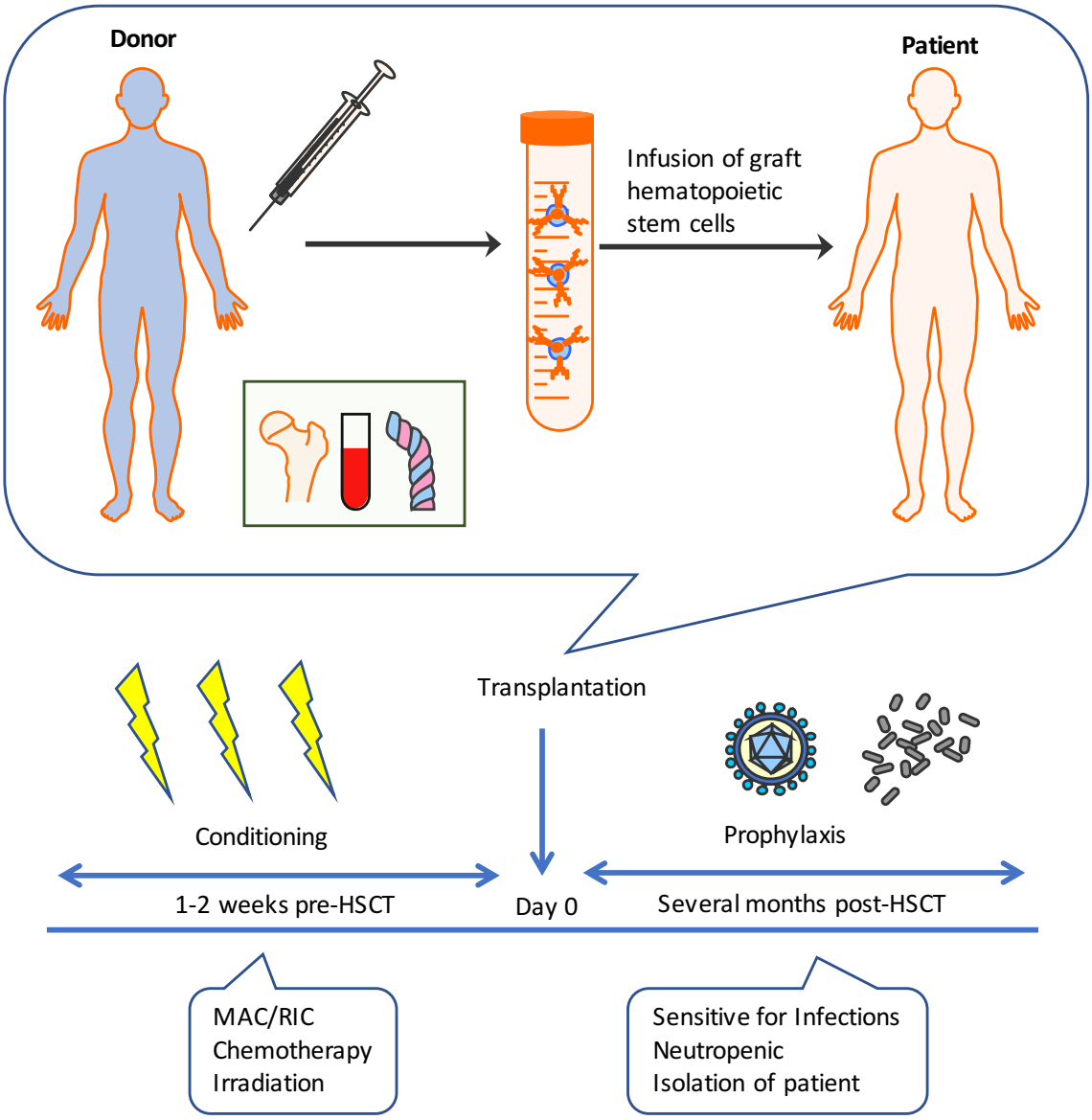

Figure 8. A schematic of the HSCT procedure. A patient undergoes a conditioning regimen for 1-2 weeks. At day 0, the day of HSCT, hematopoietic cells are harvested from the graft, be it bone marrow, peripheral blood or cord blood, and infused into the patient. During the months post-HSCT, the donor graft needs to take hold, during which the patient is at increased risk of infections and other complications. Often the patient is isolated or shielded in some form in the hospital or at home during this period.

1.2.3.1 Conditioning

Before a HSCT can be performed, the patient undergoes a conditioning regimen to prepare him/her for HSCT. The thought behind conditioning is to reduce the patient's own hematopoietic system, to suppress remaining immune cells and to eradicate the malignancy. Another argument for conditioning is the belief that space needs to be created in the patient's BM for the infused donor cells to engraft. This belief is heavily debated and remains controversial.[67]

Conditioning regimens can vary in drugs used, irradiation used and duration. Choosing the regimen depends on factors such as age and physical wellness of the patient, the underlying disorder, the experience and preference of the centre where the HSCT is performed and many more. An ideal conditioning regimen enables a quick engraftment of the donor cells, minimal toxicity for the patient and maximum elimination of the malignant cells in patients with malignancies. In broad terms, conditioning regimens can be divided into myeloablative conditioning (MAC) and reduced intensity conditioning (RIC) regimens.[68]

MAC regimens are generally considered to be more intense and harsh for patients. These regimens usually consist of high doses of chemotherapy with and without irradiation. Patients undergoing MAC become severely immunocompromised and will not survive without HSCT. They are often used in relatively fit patients who have a malignant disorder. In these cases, it is vital to eliminate as much of the underlying malignancy as possible. MAC regimens are also sometimes preferred for non-malignant disorders, as RIC regimens are associated with an increased risk of graft rejection. Moreover, patients with non-malignant disorders are quite often children who can handle tougher regimes than elderly patients. MAC regimens traditionally made use of total body irradiation (TBI) and the drug cyclophosphamide (Cy). While various combinations of drugs have been tried, the majority of MAC regimens nowadays follow the Cy/TBI, etoposide(VP-16)/TBI or busulphan (Bu)/Cy regimen.[69, 70]

RIC regimens were developed during the 1990s, and are usually considered less intense. They were developed to reduce the patient toxicity associated with MAC regimens, while retaining the beneficial effect of the graft-versus-tumour (GVT) effect of the infused donor hematopoietic cells. The development of RIC regimens enabled elderly patients as well as those with more comorbidities to potentially receive a HSCT. RIC regimens often do not include irradiation or only low amounts of fractionated TBI and reduced doses of chemotherapeutic drugs. Patients who undergo this regimen often have low levels of remaining hematopoietic cells, including defect or malignant ones, left at the day of transplantation. The infused graft will need to eliminate any remaining cells. If necessary, donor lymphocyte infusions (DLI) can be given to aid. Most RIC regimens include fludarabine (Flu). Common RIC regimens are Flu/Bu, Fly/Cy and Flu/TBI regimens.[69, 70]

1.2.3.2 Grafts

So far we have assumed that the transplanted graft came from a donor, a so-called allogeneic HSCT. It is also possible to perform a HSCT where the transplanted hematopoietic cells originate from the patient themselves, called an autologous HSCT. Autologous grafts derive from the patient themselves, hence, there are no problems with the transplanted immune system reacting in a negative manner. However, an autologous HSCT does not cure a genetic disorder, as the deficiency will still be present (in the patient/the graft). Moreover, in malignant disorders a recurrence of the malignancy after HSCT is a major risk.[71] In this thesis, the focus is on allogeneic HSCT and we will therefore not discuss autologous HSCT in any further detail.

Allografts for HSCT can be obtained from different sources. Traditionally, grafts were extracted from the BM of donors. Hence the more commonly used lay term of “bone marrow transplantation” instead of HSCT. Since hematopoietic stem cells are located in the BM, they are a perfect graft source. However, BM harvesting is an invasive, painful procedure and not without risk for the donor. For instance, donors need to undergo general anaesthesia for the operation.[72]

Nowadays, the majority of HSCTs are not performed with a BM graft, instead mobilized peripheral blood stem cells (PBSCs) are used.[61] A donor is treated for several days with granulocyte colony-stimulating factor (G-CSF), which stimulates the hematopoietic stem cells to migrate from the BM into the peripheral blood.[73] The donor then undergoes an aphaeresis for collection of the allograft. This procedure is less painful, less risky, less time consuming and no surgery nor anaesthesia is required. Moreover, PBSC grafts lead to faster reconstitution and have an enhanced GVT effect compared to BM grafts. However, PBSC grafts have been coupled to an increased risk of GVHD in some studies, though not all.[74-77] (The GVT effect and GVHD will be explained in detail in later sections.) Hence, in cases where the GVT effect is not important and the risk of GVHD should be reduced, for instance in non-malignant disorders, a BM graft is still slightly preferred.[61] However, some centres may opt to ask paediatric donors to donate PBSC and not BM, especially when donating to an adult patient, to avoid some of the difficulties in obtaining sufficient BM sample and to reduce the risk for the donor.

A third option for graft collection, umbilical cord blood (CB), has quite recently emerged, although with some mixed results.[78, 79] After birth, a substantial amount of fetal blood remains in the discarded umbilical cord. This CB is enriched in hematopoietic stem cells and if parents consent, can be harvested and stored for future use in HSCTs. These CB grafts are stored in massive CB banks and can be bought and used for HSCTs. A major disadvantage of these CB grafts is that the volume and hematopoietic stem cell content is usually quite small/low compared to BM and PBSC grafts, and can therefore often only be used for children or young adults. Some centres have attempted to perform double CB transplantations where two different CB grafts are transplanted to increase the hematopoietic stem cell content. Usually in those cases, only a single CB graft will eventually engraft and repopulate a patients BM, though in rare cases both CB grafts may survive.[80] Additionally, it is not possible to go and ask the respective donor for additional cells if necessary, as is possible in the case of conventional BM or PBSC allografts.

The possibility to ask a donor for more cells at a later time point is also one of the reasons why clinicians will investigate whether a family member could act as a potential donor. However, more importantly, family members are also much more likely to be similar for the major as well as the minor HLA histocompatibility antigens. As discussed earlier in the introduction, siblings have a 25% chance of having the same HLA-alleles. Unfortunately, it is quite likely that there is no suitable relative to act as a donor. Therefore, most current HSCTs are performed using unrelated volunteer allografts, with good results.[81] These donors volunteer themselves altruistically to give cells to patients they have never met.

In general, clinicians will try to locate a donor who matches the patient's major HLA alleles as best as possible. Some HLA alleles are thought to play a more important role than others. Therefore, centres will try to match between 8 to 12 HLA alleles. High resolution typing is usually done for HLA-A, HLA-B, HLA-C (HLA class I alleles) and for HLA-DRB1, HLA-DQB1, and HLA–DPB1 (HLA class II alleles), since these are considered to vary the most between individuals. In the end, grafts are termed to be fully matched or mismatched, when 1 or 2 HLA alleles differ.[82] A mismatched graft is sometimes the only option if no fully matched donor is found.

A relative new phenomenon is the haploidentical allograft. In this case, usually a parent or child is the donor for the patient and, as such, only half the HLA loci will match, but both for the major as well as the minor HLA histocompatibility antigens. The patient thus receives only one HLA haplotype, hence the name. The validity of this type of graft remains under debate but has shown great potential.[83] Since parents or children may act as a

haploidentical donor, the donor pool will increase dramatically. Moreover, as haploidentical donors are more likely to live in the same area as the patient, transport time will be reduced, leading to higher graft quality and viability. Thus, if haploidential allografts are proven to be a viable option, more patients will be termed eligible for HSCT. This would reduce the turnaround time for HSCTs.

Lastly, as most grafts are from volunteers, they are usually harvested in other centres and need to be transported. In some cases, the grafts are even flown in and will be underway for more than a day. Some studies have linked transport time and the environment in which the graft is placed during transport to HSCT outcome.[84-86] Thus, attempts are made to keep transport related factors between certain standards.

In the end, choosing a donor is mostly based on HLA disparity. However, many other factors, such as AB0 blood group matching, human cytomegalovirus (CMV) serology, donor and patient gender, donor age and geographic location may all influence the choice of a donor graft.

1.2.3.3 Prophylaxis

After selecting a donor and after the patient has undergone conditioning, the allograft is harvested and infused into the patient. This is referred to as "day 0". Due to the conditioning regimen, the patient is now severely immunocompromised. The new hematopoietic system needs time to reconstitute and is unable to protect the body against pathogens during this time. To support the immune system during this time, patients receive prophylactic antimicrobial drugs and specialized nursing routines to prevent excess toxicity from the conditioning (e.g. mucositis) and infections.[87-91]

Patients also receive prophylactic immunosuppressive drugs to prevent the donor's immune system from attacking healthy tissue, the complication called GVHD. This will be explained in more detail in later sections as a large part of the research presented revolves around GVHD. Some of the more common prophylactic treatments against GVHD include a calcineurin inhibitor (cyclosporine or tacrolimus) in combination with a short course of methotrexate, and in some transplant settings anti-thymocyte globuline (ATG).[92] Current treatment protocols are far from perfect and research into new prophylactic drugs and/or new combinations of drugs or supportive care is continuously ongoing.[93, 94] It is one of the main topics in many clinical HSCT trials.

1.2.3.4 Reconstitution

After HSCT, the new donor-derived immune system is impaired both quantitatively and qualitatively. It needs time to engraft and expand. During this time period a patient does not have a fully functional immune system and is vulnerable to various infections; opportunistic, community acquired or nosocomial. In a successful HSCT the donor-derived hematopoietic cells will engraft and reconstitute the patient BM, ultimately forming a fully functioning immune system. The process of forming a new immune system is called immune reconstitution and, depending on the cell type, can take months to years post-HSCT to be completed.[95, 96] Conditioning regimens, stem cell source, patient age and viral infections can also all influence the reconstitution process.[97] For instance, PBSC grafts reconstitute faster than BM grafts, administration of ATG in the conditioning regimen may delay reconstitution and elderly patients may reconstitute later as they often have an involuted thymus compared to younger patients.[98]

Cells from the innate immune system reconstitute faster than those from the adaptive arm. This is primarily because cells from the innate system do not require the same extensive rearrangement and education processes to achieve full effector functions. For instance, neutrophils engraft and repopulate to normal peripheral cell quantities just 2-4 weeks post-HSCT. In fact, neutrophil engraftment is often used as a clinical measure and indication of overall donor engraftment. NK cells usually take up to 1-3 months to return to normal levels. T and B cells from the adaptive immunity take much longer, and reconstitution can take from 3 months up to 1-2 years or longer, with persistent (or at least prolonged) deficits in terms of global immunity. T cell immune reconstitution can be thymus-dependent and thus monitored by examining thymic output by assessing TREC levels in the blood. It can also be thymus-independent, where circulating cytokines and viral infections may activate and drive expansion of memory T cells co-infused with the graft.[99] The latter mechanism usually occurs faster post-HSCT, while the first may take up to a year or more. The γδ T cells seem to reconstitute faster than the normal αβ T cells, after approximately 1-2 months. Interestingly, the TCRγδ repertoires appear to be heavily skewed towards a response directed against viral infections.[100] B cell reconstitution usually takes up to 1-2 years and is often influenced by chronic GVHD development.[90, 101-105]

In general, for most patients a fully reconstituted immune system post-HSCT can take up to 2 years. During this time, patients need to be extra careful in their daily interactions and activities to avoid infections that healthy individuals would have no problems combating. In line with this, patients will need to be revaccinated after immune cell levels have reconstituted to semi-normal levels.[90, 96]

1.2.3.5 Graft-versus-Tumour

While we do not want donor-derived immune cells to attack healthy recipient cells, we do want donor-derived cells to eliminate any remaining tumour cells. Especially in the case of RIC conditioning regimens, not all tumour cells are eradicated when transplantation takes place. This desired effect of donor cells attacking tumour cells is called the GVT or graft-versus-leukaemia effect.[106, 107]

The mechanism behind this effect is the simple way immune cells recognize the tumour cells as non-self and non-normal, triggering effector actions against such cell populations. After transplantation, clinicians must find a careful balance, stimulating GVT but not GVHD. In fact, as these are so closely related, there are many centres in which a low-grade GVHD early post-HSCT is actually desired in patients transplanted for malignant disorders.[108]

However, over promoting GVT may result in severe GVHD which can be fatal for the patient. Therefore, patients are also immunosuppressed. Administrating a heavy immunosuppressive treatment will impair the GVHD response, but also the GVT effect, and as such will increase the risk for malignant relapse. Hence, it is a constant struggle to balance GVT and GVHD and new research into enhancing GVT while reducing GVHD is ongoing.[109-113]

1.2.4 Complications

Being the only curative option for many lethal or severe disorders, HSCT remains a treatment that can come with a steep price for patients after treatment, both in terms of morbidity and mortality. A large number of complications can occur during the first months, even years, post-HSCT (Figure 9). While a large amount of research on HSCT is focused on preventing and treating these complications, and successful progress has been made over the past decades, they are unfortunately still a major part of HSCTs.[114] In fact, half of the research presented in this thesis revolves around better understanding some of these complications.

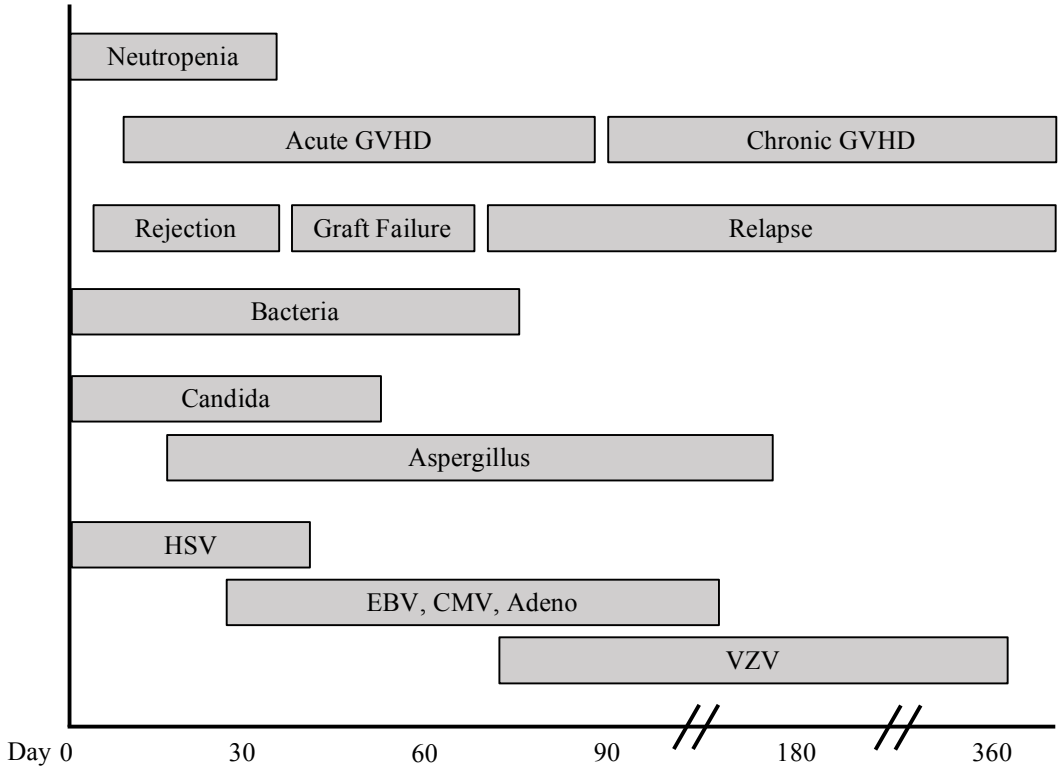

Figure 9. A schematic representation of some of the most common complications post-HSCT.

1.2.4.1 Infections

Due to previous disease treatment and conditioning regimens, patients are severely immunocompromised initially post-HSCT. During this time, patients experience neutropenia and abnormally low levels of lymphocytes in the blood. The donor-derived immune cells need time to expand. During the reconstitution, patients have an increased risk for a variety of infections. The most common infections are of viral origin, specifically human herpesviruses such as Epstein-Barr virus, CMV, varicella zoster virus (VZV) and herpes simplex virus. These are all viruses a large percentage of us continuously have in our bodies in a latent state, kept controlled by our fully functioning immune system. However, as soon as an individual is immunocompromised, these viruses "awaken" and can become a potential threat.[115-117] For this reason, patients may receive prophylactic antiviral drugs.

Bacterial infections are also a problem, though usually to a lesser degree than viral infections. This is partly because broad spectrum antibiotics are readily available and the neutrophil granulocytes reconstitute rapidly post-HSCT. However, treatment has to be

initiated quickly as patients cannot mount an effective immune response on their own early post-transplantation. This is mostly because the low levels of effector immune cells, the immunosuppression and the damage of the conditioning regimens all make life easier for the bacteria.[118-120]

Lastly; fungal infections, which can be extremely difficult to treat. They usually manifest in the lungs as fungal spores are often airborne and drawn in by breathing. With limited T cell numbers and repertoire, antifungal drugs are the only treatment option. Therefore, it is critical that these patients avoid exposure to fungi and spores during the neutropenic phase. During this time, patients are generally treated with isolation routines and are, for instance, instructed to not directly contact soil, go near construction sites, or be in buildings undergoing renovation.[117, 121]

1.2.4.2 Rejection/Graft Failure/Relapse

Another complication that can occur is rejection of the donor-derived allograft. This occurs when there are remaining recipient immune cells after conditioning which eliminate the infused donor cells. The donor-derived cells are killed and the graft is rejected by the recipient quite like how an organ can be rejected after solid organ transplantation. This leaves the patient without a fully functioning immune system. If a graft cannot take hold, the patient is at an increased risk for infections and of relapse, potentially allowing the malignancy to return in full force. The malignant cells are then even more threatening, as the conditioning effectively killed of any remaining recipient immune cells providing protection against the malignancy.[122, 123]

Fortunately, clinicians can often see a threatening relapse while it is still in its early stages. Patients can be monitored for the presence of donor- and recipient-derived cells in blood and BM, called chimerism analysis. If this analysis detects increasing amounts of recipient cells in the blood or BM over time, it indicates that recipient cells are outnumbering the donor-derived cells. In patients with a malignancy this almost always suggests a relapse of the malignancy.[124-126] However, it could also indicate graft rejection as chimerism results are relative and not absolute. For instance, if the donor-derived cells are killed by the recipient cells, the frequency of recipient cells will increase dramatically, but this is due to removal of donor-derived cells and not an increase of recipient cells. Therefore, absolute blood counts are considered when deciding if a patient faces a graft rejection or relapse.

Often, if an allograft rejection or relapse is detected before it is fulminant, steps can be taken to stop its progression and to turn the path back to engraftment of the donor cells. In those cases, the immunosuppression can be modulated or a patient can receive DLIs. These are in essence donor hematopoietic cell boosts. The DLIs will increase the number of donor-derived hematopoietic cells in the patient and can hopefully turn the tide in favour of the donor-derived immune system.[127-129] However, in some cases DLIs are unable to prevent graft rejection or relapse.[130] In such cases, the only option is to perform a re-transplantation, either with the same donor as before or with a new donor.[131-134]

1.2.4.3 Acute GVHD

Even though patients and donors are matched as well as possible for HLA alleles, a match will never be 100%. Even if the donor is an identical twin of the patient, some small histocompatibility mismatches will still be present. Unfortunately, because immune cells are trained to attack cells displaying non-self antigens, there is always a risk that the

immune cells derived from the donor graft will attack patient cells (interpreted as non-self). If donor cells attack remaining tumour cells, it is seen as a good thing. However, if the patient cells under attack are not tumour cells but are healthy, a serious complication arises. This is what happens in GVHD. If left untreated, GVHD can be lethal. Clinicians therefore face a dilemma, beneficial GVT versus harmful GVHD. How do you promote one and prevent the other? But first, we need a better understanding of the biology of GVHD.

In many ways GVHD can be thought of as an extreme form of autoimmunity. The immune system attacks the body itself. However, in GVHD, the body is not the body of the immune system, it is the body of the patient/recipient, hence, it is not an autoimmune disorder in the strictest sense of the word.

To better specify GVHD, Billingham set forth the following requirements for GVHD to occur.[135]

1. *The graft must contain immunologically competent cells.*
2. *The recipient must express tissue antigens that are not present in the transplant donor.*
3. *The patient must be incapable of mounting an effective response to eliminate the transplanted cells.*

In essence, this means that a transplanted allograft must be capable to become activated and must be able to distinguish patient cells as non-self. Moreover, the patient immune cells must be unable to mount a response towards the graft. If these 3 conditions are met GVHD can develop.

In broad terms, GVHD comes in two flavours: acute and chronic. However, the world is seldom black and white, there are many shades of grey and GVHD is no exception. I will start to explain GVHD by discussing acute GVHD (aGVHD) as this is the form that may occur first post-HSCT. Then I will discuss the in-between forms of GVHD (e.g. late onset aGVHD and overlap syndrome) to end with chronic GVHD (cGVHD) in the next section.

Acute GVHD arises within the first 3 months post-HSCT, by strict definition (Glucksberg criteria).[136, 137] Most patients start to present signs of aGVHD within 2 weeks to 2 months post-HSCT.

Table 1. Staging of aGVHD according to the Glucksberg criteria.

Grade	**Clinical symptoms**			
	Skin (maculopapular rash)	Liver (serum bilirubin)	GI-tract (mL of diarrhoea)	General clinical performance
I	<25% up to 50% of body surface	-	-	-
II	<25% of body surface up to generalised erythroderma	34 up to 50 μmol/L	500 up to 1000 mL	Mild decrease
III	25-50% of body surface up to generalised erythroderma	51 up to 255 μmol/L	1000 up to >1500 mL	Moderate decrease
IV	25-50% of body surface up to generalised erythroderma with bullous formation and desquamation	51 up to >255 μmol/L	1000 up to >1500 mL and severe abdominal pain with or without ileus	Extreme decrease

A patient can present with different grades of aGVHD severity (Table 1). Grade I manifests as skin rashes. Symptoms of grade I aGVHD are usually quite mild and might reflect an activated donor-derived immunity which is associated to a GVT effect. Hence, for patients with malignant disorders, a grade I aGVHD is often seen as a desirable phenomenon. For grade II aGVHD diagnosis, gastro-intestinal (GI) tract or liver involvement is necessary. The symptoms in these two organs remain relatively mild at this stage; slightly elevated bilirubin levels and medium quantity of diarrhoea. Grade III and IV aGVHD are the highest grades, where patients have severe symptoms from their GI tract, liver and skin.[138, 139] Acute GVHD grade III-IV is luckily rare, especially in our centre.[93, 140] However, when they do occur, these grades can be difficult to treat and are associated with severe morbidity and mortality rates.[141, 142]

Treatment of aGVHD typically consists of varying immunosuppressive regimens. As grade I aGVHD manifests as a mild skin rash, topical corticosteroids are usually administered, though in some cases systemic corticosteroids may be given too. Patients with more severe grades will often receive systemic corticosteroid treatment with varying doses. Treatment of grade III-IV aGVHD may also be done by administration of ATG, which eliminates circulating T-cells, or other immunosuppressive agents, e.g., methotrexate, infliximab and mesenchymal stromal cells.[142] A downside of treating patients systemically for aGVHD is an increased risk of infections and malignant disease relapse due to general, non-specific suppression of the immune system. As such, these patients are closely monitored during treatment. Overall, the physicians aim is to strike a balance between suppressing the immune system to prevent severe GVHD, while retaining a desired GVT effect and manageable infection rates (Figure 10).

While the severity of symptoms is crucial to diagnose the grades of aGVHD, in many cases, biopsy results of the affected organs are considered to support the clinical grading process. Biopsies can confirm diagnosis if the symptoms alone are not convincing enough.

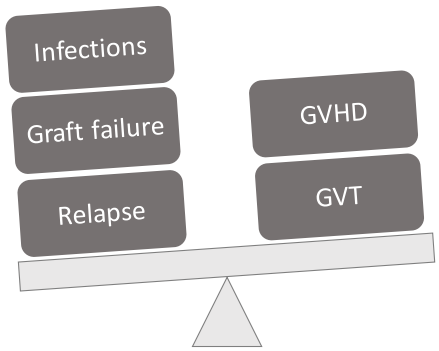

Figure 10. A careful balance between too much or too little immunosuppression is needed. Too much will increase the risk for infections, while too little will increase the risk for GVHD.

The pathophysiology of aGVHD is thought to be primarily T cell dominated, though many cellular subsets are thought to play pivotal roles in aGVHD onset and progression, as described in several reviews.[143-148] Acute GVHD pathogenesis is thought to go through three distinct stages. During the first stage, remaining patient APCs are activated by the vast tissue damage caused primarily by the conditioning regimen. This damage is due to radiotherapy, though chemotherapeutic drugs and antibiotics are also known to damage healthy patient tissue. Moreover, especially in the GI tract, tissue damage and loss of the commensal microbiota may lead to an influx of non-commensal microbes, and subsequent release of microbial products, which may further amplify APC activation.[149]

The APCs will start to display a large variety of self-antigen and varying costimulatory molecules in response to the tissue damage. This leads to the activation and proliferation of donor-derived T cells. The donor-derived T cells respond to the recipients' self-antigens as well as differences in HLA or minor histocompatibility antigens. For instance, in the case of a graft from a female donor to a male patient, the donor-derived T cells may respond to the H-Y antigens on (male) host cells.[150] Today, over 50 minor antigens that may trigger

GVHD or GVT have been identified but there is currently no matching performed for these, as this would make identifying a donor almost impossible.

The direct cytotoxic effect of the donor-derived T cells and the cytokines they produce ultimately leads to the destruction of patient tissue. This destruction leads to more damage and APC activation and the process continues in a positive feedback loop. Though not cytotoxic on their own, Th1 cells also play a role in aGVHD development. They are known to drive and maintain the CD8+ T cell response. Luckily, not only cytotoxic cells are drawn towards the inflammation. Tregs are also attracted and attempt to downregulate the ongoing process and prevent the alloreaction.[144]

Classical aGVHD is caused by the primary infusion of the donor-derived allograft. However, in a threatening relapse or rejection, a patient may be infused with a DLI to enhance the allogeneic effect in the host. In some patients, this infusion may lead to aGVHD development, referred to as DLI-induced aGVHD.[151, 152] Late onset aGVHD may also occur. This is more common in patients undergoing RIC HSCT or when immunosuppression is tapered. Overlap syndrome between aGVHD and cGVHD occurs when an aGVHD reaction does not end, but instead slowly transforms into a long-lasting cGVHD reaction. Patients with this overlap syndrome suffer from symptoms associated to both aGVHD and cGVHD. These in between forms can make it difficult to differentiate between aGVHD and cGVHD and correctly diagnose the patient.[153]

1.2.4.4 Chronic GVHD

Patients with cGVHD suffer from a prolonged attack of the donor-derived immune cells against healthy patient tissue. In cGVHD, the affected organs and symptoms are quite varied. Similar to aGVHD, skin, GI tract and liver are organs often affected, however, lungs, joints, eyes, genital areas, mouth, muscles, fascia and other sites of the body can also come under attack.[154, 155]

Traditionally, cGVHD is seen as GVHD with a late onset, often after more than a year to several years post-HSCT. However, it can occur as soon as 3 months post-HSCT. Grading of cGVHD is similar to aGVHD and is based on clinical symptoms. The NIH criteria are used to score cGVHD organ manifestations into a global classification of mild, moderate or severe grade. The symptom severity in each of the organ systems affected is scored from 0 to 3.[155] The number of affected organs and their relative classification determines the patients cGVHD grade (Table 2). For instance, patients with mild cGVHD present with mild symptoms in up to a maximum of 2 different organs, usually predominantly the skin. Patients with severe cGVHD will have severe symptoms in more than 3 organs. Involvement of the lungs is special, automatically resulting in at least moderate cGVHD. Moreover, biopsies can play an important role in diagnosis. Because so many organs can be affected in cGVHD, acquiring biopsies may be quite painful for the patient.

Unfortunately, cGVHD is common post-HSCT, occurring in around 50% of all HSCTs and even higher in HSCTs with an unrelated donor. Even more troubling is that treating cGVHD can be very difficult. Mild cGVHD, similar to grade I aGVHD, can often be treated with topical corticosteroids.[156] However, moderate and severe cGVHD often need long-term systemic corticosteroid treatment and prolonged use of calcineurin inhibitors. Unlike aGVHD where systemic immunosuppressive treatment is usually only done for a shorter period, patients with cGVHD may be treated with immunosuppressive drugs for years. In most patients the dose of immunosuppressive drugs is tapered over time as the

patient responds to the treatment. However, some patients never respond to immunosuppressive treatment. These corticosteroid-refractory patients have a very poor prognosis due to a lack of standard second-line treatment.[157] Many research groups aim at identifying treatment strategies for treating these patients.[158] Even if patients do respond to corticosteroids, those with moderate and severe cGVHD will be affected by the disease for a long period of time. Therefore, their quality of life is usually reduced.[159]

Table 2. Staging of cGVHD according to the NIH criteria.

Grade	**Scoring**			
	1 organ involved	2 organs involved	3 or more organs involved	Lung involvement
Mild	Score 1	Score 1	-	-
Moderate	Score 2	Score 2	Score 1	Score 1
Severe	Score 3	Score 3	Score 3	Score 2

While aGVHD is seen as predominantly T cell driven, cGVHD pathogenesis is seen as a combination of B and T cell involvement. However, the exact pathogenesis of cGVHD is not well understood.[154, 160] One major difference between acute and chronic GVHD is the extent of damage. While tissue damage in aGVHD is extensive and usually occurs quite fast, tissue damage in cGVHD is mostly less extensive and more fibrotic in nature. Since cGVHD damage is slower, symptoms may be overlooked for some time. Additionally, even if symptoms are quickly diagnosed, treatment is often delayed. Physicians want to be certain the cGVHD will progress if left untreated as treatment is correlated to increased risk of relapse and infections.

B cells are considered to play a major role in cGVHD. As mentioned, B cell reconstitution usually takes longer than T cell reconstitution post-HSCT. At the time of cGVHD onset, B cell levels are usually more or less back to normal. During cGVHD, B cell activating factor (BAFF), a cytokine vital for B cell survival, is elevated in patient plasma.[161, 162] Hence, potentially autoreactive B cells that would have undergone apoptosis at normal BAFF levels remain alive. These B cells may start to produce autoantibodies that target host tissues. Indeed, accumulation of such autoantibodies can be seen in cGVHD affected organs.[160, 163, 164]

The latest research also indicates a role for Th17 cells in cGVHD development, particularly of skin lesions. Tissue macrophages which are activated by the GM-CSF and IL-17 produced by the Th17 cells, will crosslink with the autoantibody coated host tissue cells. They will then start to produce cytokines, for instance TGFβ, which can explain the fibrosis often seen in the affected organs of cGVHD patients to some extent.[165, 166]

Luckily, similarly as in aGVHD, Tregs are also attracted to the site of inflammation and may play a role in dampening the response. However, as Tregs are often found at diminished levels in patients with aGVHD and cGVHD, this process is unable to stop the inflammatory response on its own. Today, some clinical trials attempt to treat cGVHD with adoptive Treg therapy, with some preliminary success.[167, 168]

As cGVHD is a common late-onset complication of HSCT with severe implications on quality of life, research in this topic is ongoing and of utmost importance.

1.2.5 Mixed Chimerism

In HSCT the ultimate aim is to successfully replace the hematopoietic system of the recipient with a donor-derived hematopoietic system. However, in some cases the recipient hematopoietic system endures, most commonly after a RIC regimen. In those cases, the term mixed chimerism (MC) is often used. In general, MC is defined as having in between 5-95% recipient-derived hematopoietic cells remaining after HSCT.

The term chimerism comes from the mythical beast Chimera from Greek mythology. The beast was a hybrid made up of several parts of different animals, though most often portrayed as a lion with the head of a goat and a snake for a tail. As patients with MC have an immune system that is derived both from the donor and the patient, the term mixed chimerism was coined. One of the first extensive studies describing MC post-HSCT was done by Ildstad et al in 1985, where they transplanted mice with a mixture of donor- and patient-derived hematopoietic stem cells. These transplanted mice were better able to tolerate skin grafts than those who only received donor-derived hematopoietic cells.[169] The first reports on mixed red cell chimerism in humans post-HSCT were published in the late 1980s.[170, 171] Since then the field has progressed and MC has also been observed in other cellular subsets.

To determine whether a patient has MC or full donor chimerism (DC) post-HSCT, the percentage of recipient- and donor-derived cells is assessed. Several techniques have been used for this and currently most are polymerase chain reaction (PCR)-based. In essence, samples from patient blood and BM are assessed for the frequency of recipient and donor-specific DNA motifs in cell subsets.[172] In our centre, myeloid, T, B and occasionally NK cells are assessed. Moreover, in general, cells from blood and from the BM are assessed separately.

Frequencies of recipient cells may also vary dramatically between cell subsets or between blood or BM samples. For instance, it is possible for a patient to be mixed in blood but not in BM, or only have donor T cells, yet have some remaining recipient NK cells. However, in general, chimerism frequency in cellular subsets tend to be quite similar.[173, 174]

The phenomenon of MC has also caught the attention of clinicians in the solid organ transplantation field, especially after the studies performed by Ildstad et al in 1985. Researchers in this field have toyed with the idea of creating a temporary mixed chimeric hematopoietic system in the patient before solid organ transplantation.[175-177] The patient would receive hematopoietic cells from the donor around the time of solid organ transplantation. If a MC situation is established, a large part of the patient's immune system would recognize the transplanted solid organ as self and would teach the other part of the immune system to ignore the non-self signal from the transplanted organ. This is the previously mentioned process of peripheral tolerance. After a couple of weeks or months the donor-derived immune cells would be rejected since the patient was not preconditioned, but hopefully by that time they will have educated the patient’s immune cells to tolerate the new organ. The patient would then theoretically no longer need immunosuppressant drugs to protect the organ from rejection. In a solid organ transplantation, patients currently need to take lifelong immunosuppressant drugs, which severely increases their risk of infections and secondary malignancies. This chimerism model for solid organ transplantation would most likely only be feasible for live organ transplants, e.g. liver and kidney transplants, as the patient would need sufficient hematopoietic cells from the same donor around the day of organ transplantation. This is often not possible in cadaveric organ transplants, such as heart or lung. Though not used in human subjects extensively as of yet, experiments have so far yielded interesting and promising results.[178-181]

While MC is seen as a potentially beneficial phenomenon in solid organ transplantation, in HSCT it is often perceived as something potentially dangerous.[182, 183] As mentioned before, if a MC develops in patients with malignant disorders, threatening relapse is often suspected. DLIs and modulation of the immunosuppressive treatment are often given in those cases to boost the donor hematopoietic stem cells to counteract relapse or donor graft rejection.[184-186]

However, in some cases, especially in non-malignant disorders, presence of residual recipient cells does not automatically indicate graft rejection or disease relapse. There have been several reports where long-term stable MC has been reported post-HSCT without any serious adverse effects. Moreover, in some instances it seems that the patients with MC are doing equally well or even better than their DC counterparts. These cases have been seen in limited cohorts only, often, though not exclusively, in siblings transplanted for non-malignant disorders.[80, 187-191]

In most cases, MC is a phenomenon that occurs shortly post-HSCT and gradually disappears after about a year. This is partly due to the general engraftment process of the donor-derived hematopoietic system and/or DLIs. In rare cases, MC persists, and is referred to as long-term stable MC. These instances have been the focal point of two of the four papers presented in this thesis (**Paper III** and **IV**). In these patients, MC is still present after more than 5 years post-HSCT. Frequency of remaining recipient cells varies largely between patients. Some present with just 5-10% recipient-derived cells, while some may have up to 90% recipient-derived cells. In the latter case, most of the patients are asymptomatic and are disease free with just a 10% fully functional donor-derived hematopoietic system.

Long-term stable MC is a fairly rare phenomenon, it only arises in a small fraction of patients and as such it is an understudied occurrence. For instance, it is unclear whether remaining recipient cells remain functional or if they are mostly anergic. And if they are functional, it is unclear whether they fulfil special niches in the immune system or whether they function as a redundant system of the donor-derived system. Moreover, why do some patients develop MC and others do not? These are just some of the mysteries that encompass MC development.

2 AIMS

The aims of this thesis were to increase knowledge on the co-existence of donor- and recipient-derived cells post-HSCT. As it is impossible to perform research on all aspects of this within a PhD project, or arguably even within a person's lifetime, focus was put on different aspects of GVHD and MC. Specifically, we aimed to:

1. Identify prognostic markers for aGVHD development.

2. Identify diagnostic markers in blood for cGVHD diagnosis.

3. Gain insights into the pathophysiology of cGVHD.

4. Gain insights into the mechanism behind long-term stable MC in non-malignant patients.

Aim 1 is discussed in **Paper I**, aim 2 and 3 in **Paper II**, and aim 4 in **Paper III** and **IV**.

3 METHODS

This section will very briefly explain the general idea behind the methods used in the research for this thesis. For a detailed protocol explaining the steps and reagents used, I would like to refer to the attached papers and the references therein.

3.1 ETHICAL IMPLICATIONS

All research performed for this thesis was approved by the Regional Ethical Review Board in Stockholm, Sweden, and performed according to the amended Declaration of Helsinki. Where required, informed consent was acquired from included patients. Patients were asked to donate blood samples and to fill in an in-house questionnaire (for **Paper IV**).

3.2 ELISA

In an enzyme-linked immunosorbent assay (ELISA), levels of a soluble factor can be measured by means of light intensity. The soluble factors are bound by an antibody coupled to an enzyme. A substrate is added which the enzyme can cleave, producing a colour change of the sample. The light intensity is dependent on the concentration of the soluble factor of interest. The measured intensity is correlated to a standard curve of known concentrations coupled to a known light intensity. Only one factor at a time can be analysed with this method. In this thesis, levels of BAFF and antibody concentrations against immunisation antigens were assessed by ELISA for **Paper II** and **IV**.

3.3 MULTIPLEX ASSAY

A multiplex assay also allows for the analysis of levels of soluble factors. In contrast to ELISA, this method uses antibody-coated beads of different sizes and can be used to analyse multiple factors at the same time. While this method is more complicated and expensive, it yields significantly more information than a single ELISA. In this thesis, 26 different cytokines and chemokines were analysed by multiplex (Luminex) for **Paper I**, **II** and **IV**.

3.4 IMMUNONEPHELOMETRY

In an immunonephelometry assay, antibody levels are measured. Samples are sorted (in the case of this thesis, sorted for isotype) and left to clump together. The sample is then passed through a light beam. The scatter of the light is measured and compared to a standard curve. The standard curve is made up of known concentrations of antibody correlated to the spread of light they produce. Immunonephelometry was used for **Paper IV.**

3.5 CHIMERISM ANALYSIS

To determine if a patient is a mixed chimera or a full donor chimera, a chimerism analysis is used. In this analysis, the percentage of remaining recipient-derived cells are determined. This is performed by analysing the sample taken post-HSCT for certain DNA motifs and comparing them to a sample from the donor and recipient before HSCT. If the sample resembles the donor sample for 100%, the patient has achieved DC. However, if some patient motifs remain in the post-HSCT sample, we can say there is MC. Based on how much patient motif is detected, the percentage of remaining patient cells can be calculated. The DNA motifs that are analysed can be as small as single nucleotide polymorphisms making the technique very sensitive. It is even more so as we can use PCR to amplify the signal of the sample to ensure enough DNA is present for the analysis prior to sequencing. Chimerism analysis was done for **Paper III** and **IV**.

3.6 WESTERN BLOT

Levels of intracellular proteins can be measured by western blot. While it is a relatively old technique, it is still used in research as it is not yet possible to perform intracellular flow cytometry staining for all intracellular proteins.
In a western blot assay, protein from cells are first separated on an SDS page gel. The proteins migrate through the gel under the influence of an electric current. Larger proteins will take longer to migrate through the gel than smaller ones, thus separating proteins by size. When the spread of the proteins of interest is deemed to be ideal for the specific analysis, the current is stopped and the gel removed. The proteins are now "blotted" onto a nitrocellulose membrane, again under the influence of an electric current, but this time from the gel towards the membrane. After this process, the proteins of interest can be stained with protein-specific antibodies for the protein on the membrane. After staining, an enzyme and substrate are added which will result in a chemiluminescent signal. The intensity and location of the signal will indicate the specific protein and its original concentration in the sample. Usually, the intensity of a "housekeeping gene" is used to calculate the relative intensity.
In this thesis, we analysed whole lymphocyte samples of patients for expression of LCK, ZAP-70, CD3δ-chain and the phosphorylation of serine and tyrosine residues for **Paper IV**.

3.7 MITOGENIC STIMULATION ASSAY

Lymphocytes can be activated in many ways, even by some chemicals. One of these chemicals is phorbol 12-myristate 13-acetate (PMA). Just up to 4 hours of exposure to PMA at certain concentrations will start the activation of T cells and the production of certain cytokines. If the cells are also exposed to a chemical called Brefeldin A, these produced cytokines will then not be excreted but instead remain in the cell. This allows the production of cytokines in each individual cell to be analysed by flow cytometry. In **Paper IV**, we performed a mitogenic stimulation with PMA and Brefeldin A for 4 hours, to assess the ability of certain cellular subsets to produce IFNγ and IL-2.

3.8 MIXED LYMPHOCYTE REACTION

In a mixed lymphocyte reaction (MLR), lymphocytes of two (or more) different individuals are mixed and left to react to each other. Usually, lymphocytes from one of the individuals is irradiated to ensure they will not proliferate. Thus, only the non-irradiated lymphocytes will be able to respond, become activated and proliferate during the reaction. As a negative control, lymphocytes from the responding individual are incubated for the same amount of time in the same setting but without the presence of irradiated lymphocytes of the second individual. The positive control on the other hand will contain lymphocytes from the first individual which will be co-cultured with a chemical known to activate the cells. Depending on the exact nature of the experiment this chemical is quite often phytohemagglutinin A (PHA). PHA is known to stimulate T cells in a broad sense.
In this thesis, we used lymphocytes from a donor-derived allograft as our responding cells, our first individual. Lymphocytes from the corresponding patient were irradiated and thus used as our second individual. Donor-derived allograft and patient lymphocytes were co-incubated in a 10:1 setting. The MLR was then left to incubate for 5 days. After 5 days, supernatant containing produced cytokines and chemokines were taken for analysis by multiplex assay. The remaining cells were harvested and stained to be analysed by flow cytometry. MLRs were performed for **Paper I**.

3.9 FLOW CYTOMETRY

In flow cytometry, the presence of intracellular and extracellular markers is analysed at a single cell level by fluorescence detection. Cells are first stained with antibodies targeting specific markers. Each antibody is labelled with a different fluorochrome. These fluorochromes will emit light at a specific wavelength after being exited by a laser at another wavelength. The fluorescence intensity indicates the amount of antibody bound to the cell. In a special flow cytometry machine, a cell sorter, we can collect cells in a separate tube based on the expression of certain markers. These sorted cells can then be used for further analysis as a "pure" cellular population.
Different fluorochromes overlap in wavelength, complicating flow cytometry analysis. This limits the number of markers one can analyse at the same time on a single cell. This number can be increased by carefully matching fluorochromes to certain markers and by increasing the number of lasers on the machine.
In this thesis, up to 9 different markers were analysed on a single cell level. A summary of the markers and their function is presented in Table 3 together with the markers used for mass cytometry. Normal flow cytometry was performed for **Paper I**, **II** and **IV**, and flow cytometry sorting was done for **Paper IV**.

3.10 MASS CYTOMETRY

Mass cytometry resembles flow cytometry in many ways. In mass cytometry, the antibodies used to stain markers on individual cells are tagged with metal isotopes instead of fluorochromes. After staining, the cells are then run through a mass cytometer, a CyTOF (Cytometry by Time of Flight) in our study, which vaporizes the cells and then uses mass spectrometry to analyse each cell. The concentration of metal isotopes per cell indicates the expression level of each marker. As the cells are vaporized during this process, it is not possible to sort the cells. A benefit of mass cytometry over flow cytometry though is that in mass cytometry the overlap between antibodies is basically non-existent as it is not dependent on analysis of emission of light. Instead, a limiting factor is the availability of metal isotopes.
In this thesis, we performed mass cytometry to analyse up to 33 markers on a single cell. A summary of the markers and their function is presented in Table 3. Mass cytometry was performed for **Paper II**.

3.11 STATISTICS

Most statistical analyses performed in the papers in this thesis were univariate non-parametric tests. In univariate tests, groups are compared on whether they differ statistically significant from each other for a single parameter. The choice was made for non-parametric tests as normality could not be assumed for all markers in all groups. The following tests were performed: Mann-Whitney-U, Kruskal-Wallis, Wilcoxon signed-rank, Friedman, Fisher Exact, Pearson's χ^2 test and Spearman's rank correlation (**Paper I**, **II**, **III** and **IV**). Post-hoc analysis used either the Bonferroni correction or Dunn's adjustment. The appropriate test was used depending on whether two or more groups were compared, whether the data was continual or categorical and whether the groups were independent or dependent on each other.
Moreover, in some instances a multivariate test was performed. Examples of these are the logistic regression (**Paper IV**) and risk factor analysis (**Paper I**). In these tests, the groups are tested for several parameters at the same time.
For most statistics the software SPSS, Statistica, R or Graphpad was used. In the case of the mass cytometry data, the software Citrus was used.

Table 3. Markers analysed by flow and mass cytometry. Some markers are also expressed by other cells than just the subset indicated.[5] Only the cell subsets for which the markers were analysed in this thesis are mentioned.

Marker	Characteristic	Cell subset
CD3	Part of the TCR complex, used to identify T cells	T
CD4	Co-receptor associated to the TCR complex, binds HLA class II	Helper T
CD5	Attenuates TCR signalling	B and T
CD8	Co-receptor associated to the TCR complex, binds HLA class I	Cytotoxic T
CD11c	Subunit of an integrin	Myeloid
CD16	Fc receptor, mediates antibody-dependent cell-mediated cytotoxicity	NK
CD19	Co-receptor associated to the BCR complex, used to identify B cells	B
CD20	Maturity marker	B
CD25	IL-2 receptor α chain	Regulatory T
CD27	Co-stimulatory receptor, part of the tumour necrosis factor receptor family	B and T
CD28	Co-stimulatory receptor	T
CD31	Adhesion molecule	T
CD38	Activation marker	B and T
CD39	Catalyses extracellular ATP, suppressive effect on immune response	Regulatory T
CD44	Adhesion marker	Leukocytes
CD45RA	Marker for memory differentiation	T
CD45RO	Marker for memory differentiation	T
CD56	Adhesion marker, used to identify NK cells	NK
CD57	NK cell marker	B, NK and T
CD69	Marker for early activation	T
CD94	Lectin, pairs to form a heterodimer with CD159, binds to HLA-E	NK and T
CD95	FAS receptor, marker for apoptosis induction	T
CD107a	Degranulation marker	T
CD127	IL-7 receptor	T
CD152 / CTLA-4	Negative regulator for activation	T
CD158b	Inhibitory KIR receptor	NK
CD159 / NKG2a	Inhibitory KIR receptor, pairs to form a heterodimer with CD94, binds to HLA-E	NK
CD161	Marker for MAIT cells, also regulates NK cell cytotoxicity	T
CD183 / CXCR3	CXC chemokine receptor, regulates chemotactic migration	B and T
CD185 / CXCR5	CXC chemokine receptor, regulates chemotactic migration	B and T
CD194 / CCR4	CC chemokine receptor	B and T
CD195 / CCR5	CC chemokine receptor, used by HIV to enter the cell	T
CD196 / CCR6	CC chemokine receptor, homing to the gut	B and T
CD197 / CCR7	CC chemokine receptor, marker for memory differentiation	T
CDw199 / CCR9	CC chemokine receptor, homing to the gut	B and T
CD278 / ICOS	Immune checkpoint protein	B and T
CD279 / PD-1	Negative regulator for activation	B and T
FoxP3	Transcription factor for regulatory T cells	Regulatory T
Granzyme B	Secreted together with perforin to induce apoptosis in target cell	NK and T
HLA-DR	HLA class II receptor, upregulated during activation	B and T cells
IgD	Immunoglobulin expressed by naïve mature B cells	B
IgM	Immunoglobulin expressed by naïve mature B cells	B
IL-2	Pro-inflammatory cytokine, expressed and produced after activation, induces T cell proliferation	T
IFNγ	Pro-inflammatory cytokine, expressed and produced after activation, activates macrophages and induced Ig class switching	T
Ki-67	Proliferation marker, absent in resting cells	B, NK and T
TCRγδ	TCR made up by a γ and a δ chain	T
TCRαβ	TCR made up by an α and a β chain, most common variant	T
TCRVα7.2	TCRαβ containing an α7.2 chain, marker for MAIT cells	T

4 RESULTS & DISCUSSION

The main focus points of this thesis revolve around increasing knowledge on the co-existence of donor and recipient post-HSCT. After the conditioning regimen, the HSCT is performed as an infusion of the allograft, i.e., the donor's hematopoietic cells. The graft needs to "settle in" and "learn" to co-exist in its new environment, i.e., the host or recipient. This complicated process of learning to adapt to the new situation and some of the difficulties patients may encounter during this process are discussed in the introduction. Several complications may arise, some of which can be life threatening. Fortunately, in most cases the patient survives with a good quality of life. The patients with a complicated HSCT course are those that could benefit from further understanding the mechanisms behind these complications. Better knowledge will result in faster interventions or new therapy strategies.

The magnitude of potential complications in HSCT cannot all be investigated within an individual PhD programme, therefore this thesis focused on aspects of aGVHD **(Paper I**), cGVHD (**Paper II**) and long-term stable MC (**Paper III** and **IV**). All three topics revolve around the interplay between the donor graft and recipient cells. In the first two topics, the allograft and recipient cells do not co-exist peacefully, as the graft attacks the recipient tissue. In the last topic, a tolerance between the graft and recipient has developed; they seem to co-exist in peace. However, what makes the well-functioning co-existence so special is that there is not just a tolerance from the graft towards the recipient tissue, there is also a tolerance from the recipient towards the graft.

For the sake of simplicity, the research will not be discussed in chronological order of when it was performed and published, instead, it will be discussed in the order of how a patient undergoing HSCT may encounter them. First, the results from **Paper I** on aGVHD will be discussed, followed by **Paper II** on cGVHD and finally, **Paper III** and **IV** which focused on long-term stable MC.

4.1 CO-EXISTENCE OR WAR?

4.1.1 Predicting Acute GVHD

As previously discussed, aGVHD is a common complication of HSCT. Low aGVHD grades are often treatable and can, in malignant disorders, be interpreted as beneficial. They indicate a strong allo-response which is required for an effective GVT effect. However, severe grades of aGVHD also occur. These higher grades are considerably more difficult to treat and are associated with high morbidity and mortality rates.

While some risk factors are known for aGVHD, it is still hard to accurately predict whether a patient has an increased risk for aGVHD at time of transplant. Some described risk factors are: HLA mismatch between donor and recipient, older patient and donor age, grafts from female donors given to male patients, the use of PBSCs, unrelated donors and ATG.[192-198] That said, there is some disparity in which risk factors may play a more determining role in aGVHD development than others. This could partly be explained by the difference in HSCT protocols and the heterogeneity of patient cohorts between centres.

Due to all of this, there is a need for more reliable and consistent risk factors or biomarkers for aGVHD development. Both predictive and diagnostic biomarkers are of interest. Similar

to risk factors, only a few biomarkers have been identified, most of which are soluble markers. A nice review was recently published by Ali *et al* which illustrates how this field has slowly grown over the last two decades.[199]

In an early example of a diagnostic biomarker study, four proteins (IL-2Rα, tumour necrosis factor receptor-1 (TNFR1), IL-8, and hepatocyte growth factor) were identified to be diagnostic for acute GVHD.[200] A follow-up study by the same group identified REG-3α levels to be diagnostic of GI tract GVHD.[201] Other studies discovered a role for certain microRNAs[202] and ST2[203] for aGVHD onset and therapy resistance, respectively. New methods like mass-spectrometry are being utilized to identify diagnostic markers, for instance, by analysing serum protein patterns to stratify patients with or without aGVHD.[204]

While diagnostic biomarkers are valuable to understand aGVHD development and progression, aGVHD diagnosis is well established by the Glucksberg criteria.[136, 137] The guidelines make use of relatively non-invasive methods (bilirubin levels, volume of diarrhoea and percentage of skin lesions) to assess aGVHD grades (Table 1). Since, severe grades of aGVHD can be difficult to treat, in an ideal world we could prevent patients from even progressing to grade II-IV aGVHD. To prevent aGVHD, we first need to accurately predict it. As such, there has been more emphasis on finding predictive markers rather than diagnostic markers in the last years.

Hence, studies have focused on finding ways to predict patients at higher risk to develop severe grades of aGVHD. An example is a study where levels of TIM-3, a marker for T cell exhaustion, was predictive of severe aGVHD.[205] This was corroborated by a later study where TIM3, IL-6 and sTNFR1 were found to be predictive of grade III-IV aGVHD development. TIM3 levels could predict aGVHD as soon as 14 days post-HSCT.[206] Another early predictor of aGVHD was found in patients transplanted with cord blood, ST2 was predictive of grade II-IV aGVHD at day 28.[207] Some studies have attempted to combine different prognostic markers to create algorithms that can predict risk for aGVHD. For instance, one study combined the previously mentioned diagnostic markers REG-3α, TNFR1, IL-2Rα and ST2, to stratify patients into low-risk and high-risk groups for onset of severe acute GVHD and therapy response. The combination of REG-3α and ST2 yielded the best results.[208, 209]

While soluble markers to predict aGVHD have received a lot of attention, probably due to their ease of use in a clinical setting, some studies have aimed to identify cellular markers. For instance, at day 30 post-HSCT, low levels of granzyme B positive Tregs was predictive for aGVHD development.[210] Invariant NKT cells have also been suggested as a potential biomarker to predict aGVHD. A reduced expansion ability of donor iNKT cells, low iNKT cell dose in grafts and a low iNKT/T cell recovery ratio post-HSCT were all positively correlated to increased grades of aGVHD.[211-213] Similar to the approach with soluble markers, another study created a five-parameter biomarker score. They combined 4 cellular subsets (CD4+ T cells, CD8+ T cells, CD4/CD8 ratio, CD19-CD21+ precursor B cells) with one soluble marker (sIL-2R) to predict aGVHD and/or overlap syndrome.[214]

Biomarker source is important. The studies described so far, all focused on identifying markers in patient material. Though more limited, some studies have also focused on identifying markers in the donor-derived grafts. For instance, two studies performed in our group identified grafts of superior viability and those containing lower frequencies of naïve CD8+ T cells to be linked to a reduced risk of aGVHD.[215, 216] One meta-analysis of 14 studies concluded that a high frequency of regulatory T cells correlated to lower non-relapse mortality and a reduced risk of aGVHD.[217] Another recent study identified a

correlation of high effector T cell frequencies and aGVHD. They also identified a protective function of high frequencies of graft CD34+ cells against aGVHD.[218] This was corroborated in a later study where patients receiving CD34+ selected grafts had a lower grade II-IV aGVHD incidence.[219] Finally, a third study from our group, and the pilot study for **Paper I,** identified, among other subsets, that reduced frequencies of γδ T cells were linked to grade II-IV aGVHD development.[220]

While a few potential biomarkers, both predictive and diagnostic, have been identified and are being studied in detail, there is still no clear-cut biomarker that can easily predict aGVHD. Moreover, useful biomarkers in one centre may not be the same in another, due to the differences in HSCT protocols. Therefore, our aim for **Paper I** was to identify diagnostic markers for aGVHD development that could be utilized in our clinical setting. Hence, the methods had to be simple and easy to implement in a routine laboratory.

Paper I focused on elucidating predictive biomarkers for aGVHD, with a focus on cellular markers. We analysed grafts from more than 100 donors and correlated them to primary aGVHD outcome in the corresponding grafted patients. Development of aGVHD after DLI infusion was not taken into account in aGVHD grading and grouping the results.

Patients were split into two groups, one group that developed either no aGVHD or grade I aGVHD against a group of patients with grade II or higher aGVHD. This stratification was based on clinical relevance. While it would have been interesting to analyse patients with higher grades separately, unfortunately, such an analysis was not feasible due to the limited number of patients with grade III-IV aGVHD.

There were no differences observed in clinical parameters between the grade 0-I and grade II-III aGVHD patient groups. All grafts were extensively assessed by flow cytometry and the immune phenotypes of the two patient groups were compared. Several cellular subsets of interest emerged. Of particular interest were the reduced frequencies of CD4+, CD27+, CD28+ and CD127+ T cell subsets and the increased frequencies of CD8+ and programmed cell death 1 (PD-1)+ T cell subsets in the patient group with grade II-III aGVHD.

While patients with grade II-III aGVHD received grafts with a more pronounced shift towards CD8+ T cells than patients with grade 0-I aGVHD, all grafts contained more CD8+T cells than CD4+ T cells. The normal distribution of blood T cells is the opposite, with more CD4+ T cells than CD8+ T cells. The inverted CD4/CD8 ratio is a common feature post-HSCT.[221-223] However, this inverted ratio is seemingly already present in the grafts, indicating that this is not purely a result of the reconstitution. The shift in CD4/CD8 ratio was seen in both BM and PBSC grafts, so it seems unlikely that the stem cell extraction itself accounts for this shift. Perhaps the graft processing before transplant favours survival of CD8+ T cells over CD4+ T cells. Data on immune phenotyped grafts at different time points from collection to infusion has not been published to our knowledge. Hence, it would be interesting to determine whether graft procurement and handling affects the cellular immune phenotype and the impact that may have on the transplant outcome.

As mentioned, T cells positive for CD28 or CD127 were found at lower frequencies in grafts given to patients who later developed grade II or higher aGVHD. Both markers are involved in T cell activation. CD28 is the costimulatory signal required for primary activation of a T cell and CD127, the IL-7 receptor-α chain, is central in T cell proliferation. From the reduced expression of CD28 and CD127 we can thus speculate that the grafts given to grade II-III aGVHD patients were of a more naïve memory phenotype.

This finding is in line with several other studies where a positive correlation existed between naïve memory T cells and aGVHD.[216, 224-226]

However, lower CD127 expression could also be interpreted as a shift from naïve to effector memory phenotype of the grafts. After activation, CD127 is downregulated upon maturation as effector memory T cells express hardly any CD127. Additionally, since effector memory T cells no longer need co-stimulation, this would fit the reduced CD28 expression as well. It would also fit the observed increased expression of PD-1. While PD-1 is mostly seen as an exhaustion marker, it is constitutively upregulated on activated cells as a negative feedback mechanism.[227] Moreover, the reduced expression of CD28 and increased expression of PD-1 nicely complement each other. These markers are known to have mostly opposite functions in the costimulatory/inhibitory aspect of the T cell activation cascade.[228]

When analysing the grafts for memory phenotype (by CD45RO and CCR7 expression) there were no statistically significant differences between the memory subsets of the patient groups. However, we did identify a trend for a shift from naïve towards a more effector memory phenotype in the grafts given to the grade II-III aGVHD patient group.

It is interesting to consider the effect of administering grafts with a more pronounced effector memory phenotype. Effector memory T cells do not require co-stimulation and can thus be triggered to react and destroy simply by receiving distress signals from the tissue. However, preventing co-stimulation by blocking CD28, seems to still be able to impair and delay effector memory T cell response to a second infection to some extent. The exact mechanisms are not entirely clear yet and, as such, this is still somewhat controversial.[229, 230] Nevertheless, as HSCT patients experience extensive tissue damage due to the conditioning regimen, there will be plenty of danger and warning signals for the effector memory T cells to react to. Add to this that the donor cells will detect the recipient tissue cells as non-self, since all patients have some degree of mismatch in either the major or minor histocompatibility antigens, and you have a recipe for immunologic disaster.

Even though the results of the cellular subsets found in **Paper I** are interesting on their own, it would be even more interesting if these markers had some actual predictive value. Hence, we performed a risk factor analysis and created a receiver operating characteristic (ROC) curve (Figure 11). The risk factor analysis may be easier to comprehend but it only looks at the data in a binary manner. An ROC curve calculates for each possible threshold the true positive rate versus the false positive rate and visualizes them on a graph in the form of a curve. The more the curve hugs the upper left corner, the better the combination of markers is at accurately predicting the binary outcome, development of aGVHD in this case. An ROC curve will also give an area under the curve (AUC) value for each model tested. This allows for the selection of the best model by comparing AUC levels.

We started with the risk factor analysis to assess whether the above-mentioned subsets would equally contribute to the risk of grade II or higher aGVHD. As CD4 and CD8 expression are related, we chose to include only one of these markers. The choice was made for CD4 expression, based on the higher availability of CD4 fluorescence markers and the simplicity of gating CD4 over CD8.

Patients were assessed to have a risk factor for each marker if the grafts presented below median frequencies of CD4, CD27, CD127 and CD28 expressing T cells and an above median frequency of PD-1 gated T cells. Hence, the patients were divided in different groups depending on the amount of risk factors.

The combined effect of all five risk factors led to a grade II-III aGVHD incidence of 76%, while having four risk factors or less led to an incidence of between 19-37%. We then aimed to identify which risk factors had the most pronounced effect on the grade II-III aGVHD incidence. The combination of the risk factors PD-1 and CD127 had an incidence of 67%, which, besides including all five risk factors, led to the highest incidence prediction. These two markers had the greatest power to predict aGVHD.

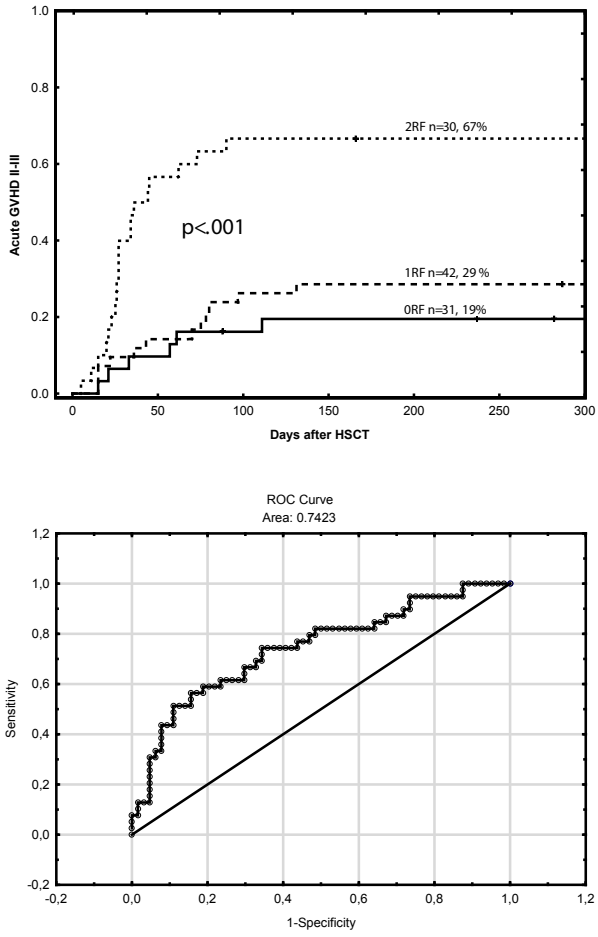

Figure 11. A Risk Factor analysis and ROC curve of the impact of PD-1 and CD127 on aGVHD development.

An ROC curve was plotted for these two markers to assess the predictive value of the markers. The combined effect of PD-1 and CD127 expression on total T cells led to an AUC value of 0.74, which indicates a strong predictive value. However, a second cohort will be needed to verify these results. If these results hold true, it would be particularly interesting as these are merely three markers (PD-1, CD127 and CD3) that would need to be analysed during graft processing. As most centres already assess graft viability by flow cytometry, adding one more sample should be possible. As a result, clinicians will receive information not only on graft viability but also an aGVHD risk assessment at the time of transplantation. This knowledge can positively influence patient monitoring and treatment post-HSCT. For instance, when early aGVHD symptoms arise, a patient at increased risk could be treated earlier or more intensively, while a low risk patient could be treated accordingly. Unnecessary treatment can thus be avoided, resulting in less infections, higher quality of life and reduced costs for the clinic.

The six above mentioned T cell markers were not the only markers that differed between the grade 0-I and grade II-III aGVHD patient groups. We also identified differences in a MAIT subset and a B cell subset (Figure 12).

As mentioned in the introduction, MAIT cells are a specialised subset of T cells usually found in low frequencies in the blood. They are, as the name already suggests, usually associated to the mucosa, and are thus more abundant in the skin, liver and gut.[27, 231] As these tissues are also the sites where aGVHD occurs, the potential role of these cells in aGVHD is interesting.

MAIT cells are defined as T cells that are double positive for TCRVα7.2 and CD161. Moreover, most MAIT cells do not express CD4. As a mucosal-associated cell, most MAIT cells express a variety of homing receptors for varying mucosal tissues. Classic examples of these are CCR6 and CCR9.[27, 28, 232]

The MAIT subset that differed between the two patient groups was a CCR9+ CD4- CD8+ MAIT cell. This subset was found in lower frequencies in grafts given to patients with grade II-III aGVHD (Figure 12A). Since patients who did not develop high grades of aGVHD received higher frequencies of this MAIT cell subset, we can hypothesise that this subset might have a protective role in aGVHD development. Perhaps these cells can dampen the immune response in some way. For instance, by clearing up the damage associated to the tissue, negating the distress signal sent out by the tissue to the innate and adaptive immune mechanisms.

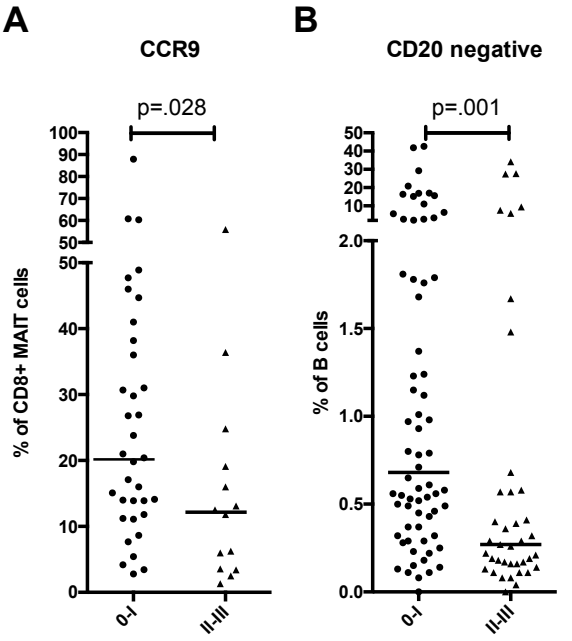

Figure 12. Frequency of a MAIT and B cell subset, comparing aGVHD grade 0-I and grade II-III aGVHD patients.

A specific B cell subset also differed in frequency between the two patient groups. The vast majority of human B cells express the cellular marker CD20 (also known as B-1), only pro-B cells and plasma cell do not.[233-235] These two cellular subsets respectively represent the beginning and the end phase in B cell maturation. Both subsets also reside in the BM. Coincidentally, the drug rituximab, often used to treat CLL, targets CD20 on B cells, resulting in the death of all CD20+ B cells.[236, 237]

In this study, CD20- B cells were found at lower frequencies in the graft in the grade II-III aGVHD patient group (Figure 12B). These CD20- B cells are not those that are activated by CD4+ T cells, since these still need to mature or are producing antibodies against previously encountered pathogen. We could speculate that lower frequencies of these cells could protect the patient against CD4+ T cell driven inflammation.

Due to restrictions in the flow cytometric antibody panel, we could not assess whether these CD20- B cells constituted pro-B cells or plasma cells. Since both cell subsets reside in the BM and since we have a mixed patient cohort of BM and PBSC-derived grafts, we analysed whether this difference in CD20- B cell frequency was not caused by a difference in graft source. As expected, irrespective of aGVHD development, CD20- B cells were more abundant in the BM grafts than in the PBSC grafts ($p<.001$). To assess the potential impact of this finding we analysed whether the CD20- B cell frequency differed between the aGVHD patient groups for each graft source separately. Interestingly, CD20- B cell frequency did not differ between the aGVHD patient groups in the BM grafts, but did differ in the PBSC grafts ($p<.001$). Hence, the CD20- B cell frequency difference observed in Figure 12B between the aGVHD patient groups was due to a difference in CD20- B cell frequency in the PBSC grafts.

The CD20- B cell frequencies are low, especially in the PBSC grafts, and may not have a practical application in the clinic for predictive purposes. However, they may be important to understand aGVHD development. Identifying whether these CD20- B cells are pro-B cells or plasma cells could be imperative. For instance, pro-B cells will mature and proliferate further in the patient and may thus have a larger effect than we can perceive at the moment.

Investigations into the effect of graft source stimulated us to look into whether the graft source could influence the previously mentioned markers. PBSC grafts have been G-CSF stimulated, which may affect the activation phenotype of the immune subsets. Hence, we performed a similar analysis for the six markers (CD4, CD8, CD27, CD28, CD127 and PD-1) to assess the distribution of these subsets over the graft source regardless of aGVHD grading. No differences between the graft source groups for subset frequencies could be observed. Hence, the differences we saw in marker subsets between the patient groups are true differences observed in the donor-derived graft and not due to the G-CSF stimulation.

In addition to immune phenotyping the donor grafts, we also assessed the graft functionality and reactivity between the grade 0-I and grade II-III aGVHD patient groups. To achieve this, we performed MLRs by incubating donor graft-derived cells in three different settings. For the first setting (negative control), we left donor graft cells un-stimulated in complete medium. This would illustrate baseline activity. In the second setting (positive control), we stimulated donor graft cells with PHA. PHA activates all lymphocytes, though the exact mechanism is not completely understood.[238] This illustrates the maximum activity possible for the donor graft cells and functions as a quality check of the graft. Finally, the third setting was created to try and mimic the *in vivo* setting in the patients in aGVHD development. Patient lymphocytes were irradiated to ensure they would not proliferate and survive the incubation. Moreover, the irradiation damages the cells similar to the damage made by the conditioning regimen. The patient lymphocytes were then added in a 1:10 ratio to the donor graft cells. Hence, we seeded 10× more donor graft cells than patient lymphocytes.

The method has some drawbacks. First off, in an MLR we test the reactivity of graft cells against patient lymphocytes. This is in contrast to aGVHD which is usually directed at epithelial cells, not lymphocytes. However, as it is not feasible to acquire skin, gut and/or liver biopsies of all patients, especially before HSCT, an MLR is an accepted method to model donor-patient reactivity in an *in vitro* setting.[239-241] Additionally, in a patient, ratios of patient and donor cells are actually reversed. There are vastly more patient epithelial cells than donor cells post-HSCT. Therefore, perhaps it would have been truer to reality to seed donor and patient cells in a 1:1 ratio. Adding even more patient cells could blur the results at the end of the incubation as all patient cells will die due to the irradiation. Patients undergoing HSCT are usually not in the best of shape and have undergone other therapies prior to becoming eligible for HSCT. As a result, these patients are usually lymphopenic. We tried to acquire as much lymphocytes as possible using blood samples taken before the start of conditioning regimen. We feel that the 1:10 ratio used, although not ideal, was the best option under the circumstances. Performing MLRs are thus not without its complications, but they are one of the most used *in vitro* aGVHD models for patient material.

We performed an MLR for 80 patient-donor pairs. Some patients and donors were excluded due to low patient lymphocyte numbers or logistic issues. The MLRs were assessed by flow cytometry. The choice to focus on T cells was done for several reasons. One reason is the fact that aGVHD is described to be a T cell driven disease. Secondly, due to low patient lymphocyte numbers, we were limited in the number of flow cytometric panels. Lastly, most NK and B cells do not survive the incubation without proper addition of relevant cytokines, nutrients or specific stimulator cells.[242]

Analysis of blast formation, indicative of T cell activation, in the three different settings after the MLR, demonstrated that the donor graft cells in the PHA setting were extensively activated while the unstimulated and 1:10 settings resembled each other with few blasts.

Moreover, frequencies of some of the main cellular subsets of the immune system, total T cell, CD4+ T cell, CD8+ T cell, regulatory T cell and maturation status, were similar between the patient groups for all three MLR settings. Looking slightly more in depth to several cellular subsets did yield some differences between the grade 0-I and II-III aGVHD patient groups for the 1:10 and PHA setting. As the 1:10 setting is deemed to be the *in vitro* model for aGVHD development, we will focus the discussion of cellular subsets based on this setting.

In the 1:10 setting, there were several interesting statistical differences in CD4+ T cell subsets between the grade 0-I and II-III aGVHD patient groups. CD4+ T cells positive for activation markers CD94, CD56 and CD69 and positive for degranulation marker CD107a, were found at increased frequencies in grafts given to patients who later developed grade II-III aGVHD. Frequencies of CD4+ T cells positive for exhaustion markers PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) were also increased in these patients. This indicates a more recently activated cytotoxic phenotype in those grafts after being exposed to recipient lymphocytes. It is possible that the grafts given to these patients had a lower activation threshold towards recipient antigens. This phenotype was not found to be statistically different in the CD8+ T cells. We can hypothesise that CD4+ and CD8+ T cells have differing activation kinetics[243] and, as such, by looking at only a single time point we may have missed the optimum for (one of) these subsets.

Due to patient sample availability, we were limited to assess the immune cell phenotype at one time point. In a future study, it would be beneficial if the MLRs could be analysed at several time points. This would either require larger patient samples, which will be a challenge as these patients already give blood for a range of clinical tests, or we would need to reduce the number of flow cytometric panels. If we can reduce the number of panels by a factor of five, we could analyse the immune phenotype on a daily basis, instead of just once at the end of the 5 day incubation. Perhaps this would allow us to better assess the kinetics of recipient antigen activation.

We also collected supernatant at the end of the 5 day MLR. We hypothesised that even if this time point was not optimal to detect a maximum response, we would still observe activation in the form of accumulated soluble markers in the supernatant. Hence, we analysed the supernatant for 26 cytokines and chemokines. Strikingly, the only cytokine to truly emerge was tumour necrosis factor (TNF)α (Figure 13A). It was found at increased levels in the supernatant of MLRs from grafts given to patients who later developed grade II-III aGVHD. This was only observed in the unstimulated setting. We speculated that this TNFα level increase may be seen as a baseline activity in the donor-derived graft cells. Unfortunately, we were unable to assess cytokine levels in the actual original graft samples as these were treated slightly different and given to us in varying concentrations. Additionally, to standardise the allograft samples, we performed a density gradient centrifugation on all samples. Cytokines would have been washed away. Given the circumstances, the cytokine levels in the unstimulated settings of the MLRs are thus the best substitute we have for baseline cytokine activation of the graft samples.

The increased TNFα levels in the unstimulated setting intrigued us, so we next assessed the TNFα levels in the patients before and after HSCT. First, we analysed how each patient group varied in TNFα levels over time. For both patient groups, we saw a marked decrease in TNFα between before conditioning and day 14, and a marked increase in TNFα between day 14 and 1 month post-HSCT. The overall pattern of TNFα levels was the same between the patient groups (Figure 13B). However, when we analysed TNFα levels between the patient groups per time point, we observed a significant increase in TNFα levels in patients

who later developed grade II-III aGVHD before conditioning and at day 0. At day 14 and 1 month post-HSCT, differences in TNFα levels between the patient groups disappeared (Figure 13C). As a general and early-induced inflammatory cytokine, increased levels of TNFα as an indicator of increased risk of grade II-III aGVHD is to be expected. TNFα production has also been shown to be associated to complications post-HSCT such as severe aGVHD and interstitial pneumonitis.[244-247] Moreover, a study in mice indicated a specific role for the soluble form of TNFα over the membrane bound form in GVHD development. By blocking the ability of murine donor T cells to form soluble TNF, mice developed less GVHD but retained the GVT effect.[248] However, these associations are on their own not strong enough to act as a reliable predictive marker in the clinic.

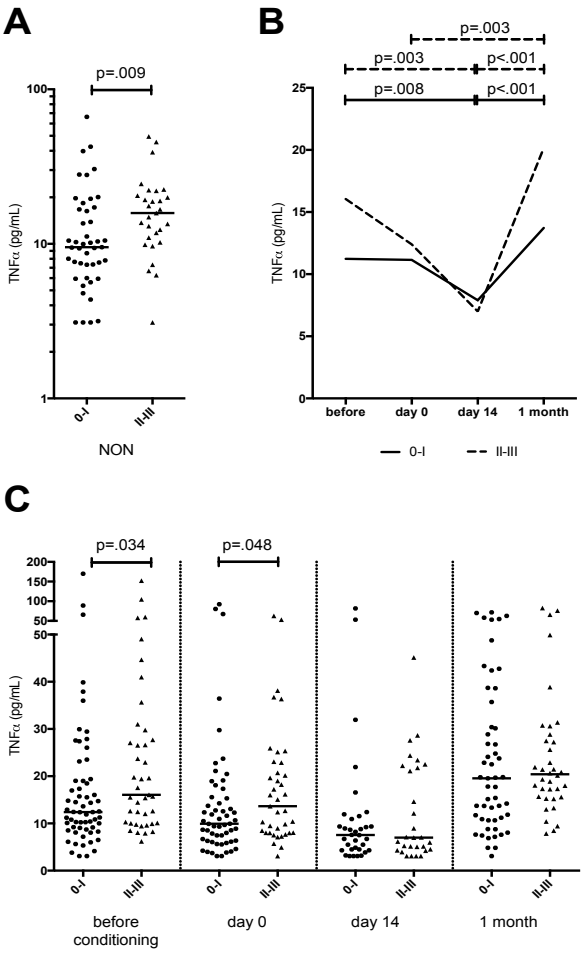

*Figure 13. TNFα levels in patients **(A)** after a 5 day unstimulated incubation, **(B-C)** before conditioning, at day 1, 14 and 1 month post-HSCT.*

We hypothesise that TNFα in the patient alone is insufficient to predict aGVHD, however, combining TNFα levels in the graft and recipient may be more reliable. In this study, patients who developed grade II-III aGVHD not only had elevated peripheral blood TNFα levels before conditioning and at the time of HSCT, they also received grafts with a potential increased baseline TNFα production. This combination may be an important catalyst of aGVHD development. We theorise that grafts with a potential increased TNFα production have a low activation threshold. These cells are then infused into a host milieu with even more elevated levels of TNFα. Subsequently these cells now come into contact with a recipient epithelial cell, expressing unfamiliar histocompatibility antigens and a lot of costimulatory molecules, resulting in them delivering the immunologic attack, i.e., GVHD. This hypothesis needs further investigation.

To conclude, while it is difficult to find predictive biomarkers in the diverse patient population undergoing HSCT, there are some cellular markers that may fulfil such a function. As we demonstrated, PD-1 and CD127 expression on T cells may have a predictive value for severe aGVHD. It should be possible to implement staining and analysis for these cellular subsets in clinical routine. Additionally, analyses of TNFα levels in the grafts and patient peripheral blood should also be possible to do in a routine manner. Only then, after a prospective trial in a large cohort can we truly ascertain whether these biomarkers hold merit as predictors of aGVHD.

We feel that while the results obtained in this study are promising, there are a number of additional steps required to further elucidate and confirm these findings. We will discuss some of these in the "concluding remarks and future aspects" section.

4.1.2 Quest for Biomarkers Chronic GVHD

While aGVHD is an important and debilitating complication, cGVHD is equally important from a patient's perspective. They may suffer from cGVHD for many years having an enormous impact on quality of life. The pathophysiology of cGVHD is less well understood than of aGVHD, as also discussed in the introduction. Additionally, diagnosis of cGVHD can be difficult and often requires biopsies. Depending on the organ affected, biopsies may be painful and/or dangerous. Hence, we aimed to identify potential diagnostic cGVHD markers in peripheral blood and potentially gain some insights into the pathophysiology.

Several studies have tried to identify risk factors for cGVHD development. Several risk factors have been known to be overlapping with aGVHD. For instance, HLA disparity, older patient age, grafts from female donors given to male patients, the use of PBSCs and ATG. Some other described risk factors are; prior aGVHD, splenectomy and CMV seropositivity.[192, 196-198, 249-252]

In the last decade, more research has focused on identifying diagnostic and predictive biomarkers. As cGVHD can still be difficult to diagnose correctly, especially without performing biopsies, I focused on diagnostic biomarkers in **Paper II**.

Similar to aGVHD, most published data on cGVHD concerns soluble markers in peripheral blood of patients due to accessibility. Several studies identified BAFF levels to be increased in patients with cGVHD.[161, 253-255] Two of these studies also observed increased levels of sIL-2Rα and soluble CD13 to be linked to cGVHD.[253, 254] Related to BAFF, increased levels of a proliferation-inducing ligand (APRIL), known to be important in B cell isotype switching, was positively correlated with severe cGVHD and increased plasmablast frequencies in cGVHD patients.[256] Additionally, high levels of CXCL9[254, 257, 258], ST2[257, 258], TNFα[259 260] and soluble MICA[261] were also associated to cGVHD. On the other hand, high levels of IL-15 correlated to a reduced risk of cGVHD.[262]

There are studies that have focused on identifying cellular biomarkers for cGVHD. Several examples of promising results are the following. Some studies have shown that low NK cell doses in grafts can be protective of cGVHD development.[263, 264] This was corroborated in a slightly newer study, which identified levels of total NK cells and CD152+ (also known as CTLA-4) T cells to be negatively correlated to cGVHD.[259] Other studies identified a role for B cells in cGVHD. Patients with cGVHD had lower total B cell counts, but higher frequencies of IgD+ B cells and pre-germinal centre B cells than those without cGVHD.[255] Another study on B cells identified higher frequencies of $CD38^{hi}$ plasmablasts in patients with ongoing cGVHD.[265] In line with his finding, one study observed reduced frequencies of Tfh cells, though they were more activated and skewed towards an Th2 and Th17 phenotype in cGVHD patients. This, coupled with an increased level of CXCL13, led the authors to speculate that the Tfh cells migrated toward the secondary lymphoid organs to activate and mature B cells, increasing the cGVHD severity.[266] Another study identified a correlation between low levels of monocytes and high levels of CD34+ cells in the graft with cGVHD development.[218] However, a more recent study observed a low incidence of cGVHD with patients receiving CD34+ selected grafts.[219] Lastly, Th17 cells have also been a target of research in cGVHD. A higher frequency of Th17 cells was seen in patients with active cGVHD.[165] Since then, Th17 involvement in cGVHD has been studied in more detail. A recent study focused on how Th17 frequencies in the liver affects cGVHD. They observed an increased infiltration of Th17 in the liver of patients with hepatic cGVHD.[166] Th17 cells are especially interesting as they have been linked to diseases such as systemic sclerosis[267, 268], which is characterized by extensive tissue fibrosis somewhat resembling the fibrosis observed in cGVHD.

Despite all these studies, few biomarkers have made their entrance into the clinical setting and are currently used to diagnose cGVHD severity. Hence, we aimed to identify markers that could potentially be used as diagnostic tools or could help us understand cGVHD pathophysiology better in our patients.

For **Paper II** we started by collecting samples from patients suffering from varying grades of cGVHD; mild, moderate and severe (Table 2). All patients were retrospectively scrutinised for cGVHD grade by studying the medical records around the time of blood donation. Since the NIH guidelines to score cGVHD were implemented as recently as 2014, we wanted to make sure all patients were classified in the same manner. As a control group, we collected samples from patients who did not suffer from cGVHD. All patients were at least 1 year post-HSCT and none were suspected of overlap syndrome.

To ensure that we would not burden these patients unnecessarily, patients were asked to participate in the study during a routine check-up. As we collected samples from the patients, we identified a considerable imbalance in intake of immunosuppressive drugs between the different patient groups. Patients without cGVHD or mild cGVHD had not received long-term systemic immunosuppressive treatments, while patients with moderate and severe cGVHD had.
As immunosuppressive drugs alter the immune-phenotype, the decision was made to only compare patients without cGVHD to mild cGVHD patients and moderate to severe cGVHD patients for analyses done on blood samples taken at inclusion time. For parameters around the day of HSCT, we compared the four patient groups to each other as at that time point they were still similar.

In total, 68 patients were included, divided over two study cohorts. The detailed patient characteristics can be found in the tables of **Paper II**. The four patient groups were similar for most clinical characteristics apart from anti-T cell antibody treatment. However, this difference was only seen when comparing patients without cGVHD and mild cGVHD versus patients with moderate or severe cGVHD. There was no difference when comparing patients without cGVHD to mild cGVHD and when comparing patients with moderate to severe cGVHD. As we compared patients only in this latter manner for the rest of the paper, we felt the difference in anti-T cell antibody treatment, though interesting, was not a potential confounder for our analysis. Additionally, we found a positive correlation between aGVHD development and cGVHD development. This was an expected outcome, as aGVHD is a known risk factor for cGVHD.

The patients were stratified into two cohorts. The first cohort of 53 patient samples was analysed by conventional flow cytometry, ELISA and a soluble marker multiplex assay. Moreover, 40 patients of this cohort were analysed by mass cytometry to identify novel cellular subsets correlated to cGVHD severity. The second patient cohort consisted of 37 patients of whom 15 patients were new to the study and 22 had been included in the first cohort. The samples from the second cohort were analysed by flow cytometry to confirm the findings of the mass cytometry in a more routine, clinically applicable method.

Ultimately, for a diagnostic marker to be successful it has to be a marker that is relatively easy to assess on a large and fast scale in a routine laboratory. Hence, we started by looking at soluble markers. We performed a 26 cytokine/chemokine multiplex assay on the patient plasma samples from the first cohort. Among the analysed cytokines were IL-2, IL-15 and TNFα, which have been linked to cGVHD severity, as discussed earlier. However, no differences in these cytokines or the other 23 cytokines/chemokines could be observed in our cohort.

One of the most consistent soluble biomarkers identified with cGVHD is BAFF. In our cohort, while we could not identify a difference in BAFF levels directly, we could identify a significant difference in BAFF/B cell ratios between patients without cGVHD and mild cGVHD and a trend toward significance between moderate and severe cGVHD patients (Figure 14). In line with previous studies, BAFF and the BAFF/B cell ratio were increased in patients with a higher grade of cGVHD. Interestingly, there were no differences in total B cell or memory B cell frequencies between the groups.

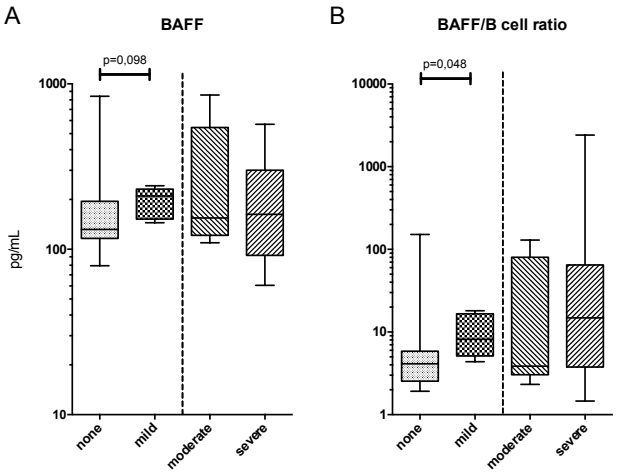

Figure 14. Serum protein phenotype. BAFF levels and BAFF/B cell ratio differences between cGVHD patient groups.

It is remarkable that we could only detect a difference in BAFF levels in patients with mild cGVHD compared to those without cGVHD. We have hypothesised that the high dose of immunosuppression given to patients with moderate and severe cGVHD has had a levelling effect on the immune phenotype, as described in a previous paper on BAFF levels.[161] In fact, in the other analyses we performed, most striking differences were observed between patients without cGVHD and patients with mild cGVHD, while differences between patients with moderate and severe cGVHD were often less pronounced. Though this could partly be due to the difficulty in grading patients as either moderate or severe, most of the effect is probably due to immunosuppression.

Similar to the previously mentioned study[161], we assessed whether it would be possible to analyse the impact of immunosuppression on BAFF levels within the moderate and severe cGVHD group. This was not possible as only one patient in this group did not receive systemic treatment at the time of inclusion. Therefore, we can only speculate on the effect of immunosuppression on the levelling of the immune phenotype between moderate and severe cGVHD patients.

Even though it would be most practical to identify a soluble marker as diagnostic tool for cGVHD, we also looked at cellular phenotypes. We analysed a large variety of T, B and NK cell subsets by flow cytometry. No differences between the patient groups for the more common main cellular subsets, e.g., total T cells, CD4+ T cells, CD8+ T cells, Tregs, memory differentiation subsets, B or NK cells could be detected. Hence, we started to look in depth into rarer subsets.

One of the subsets that differed between the patient categories were MAIT cells. As mentioned in the introduction and also the discussion section on **Paper I**, MAIT cells are a relative small cellular subset in blood. Moreover, they are a quite new discovery. Before the discovery of the TCRVα7.2 antibody, the closest researchers could get to MAIT cells was by identifying them as T cells producing IL-17 and displaying CD161 and CCR6. However, Th17 cells also fall under this umbrella of cells. There have been studies that looked at these subsets and correlated them to cGVHD, though with conflicting results. Both a reduced IL-17 producing T cell frequency[269] and an increased Th17 cell frequency[165] in patients with cGVHD have been observed. Hence, studies performed on MAIT cells before the TCRVα7.2 antibody became available are difficult to compare to current research. However, they do implicate Th17 or MAIT cells in cGVHD development.

In our study, we observed reduced frequencies of MAIT cells in patients with increasing cGVHD severity (Figure 15), similar to one of the aforementioned studies.[269] This was true regardless of whether the MAIT cells were CD4-, CD4-CD8+ or CD4-CD8-. Similar to the BAFF results, the difference between the groups was most pronounced between patients without cGVHD and mild cGVHD. As MAIT cells are drawn towards the mucosal areas of the human body, we speculate that the MAIT cells migrate towards inflamed mucosal sites in patients with more severe cGVHD. While we did not perform patient biopsies to confirm this, studies on patients with inflammatory bowel disease and ulcerative colitis observed reduced MAIT cells in the blood and increased MAIT cell frequencies, or CD161 expression, in the inflamed gut tissue.[270-272] In a future study, it would be interesting to see if MAIT cell frequencies are indeed increased in cGVHD affected tissues.

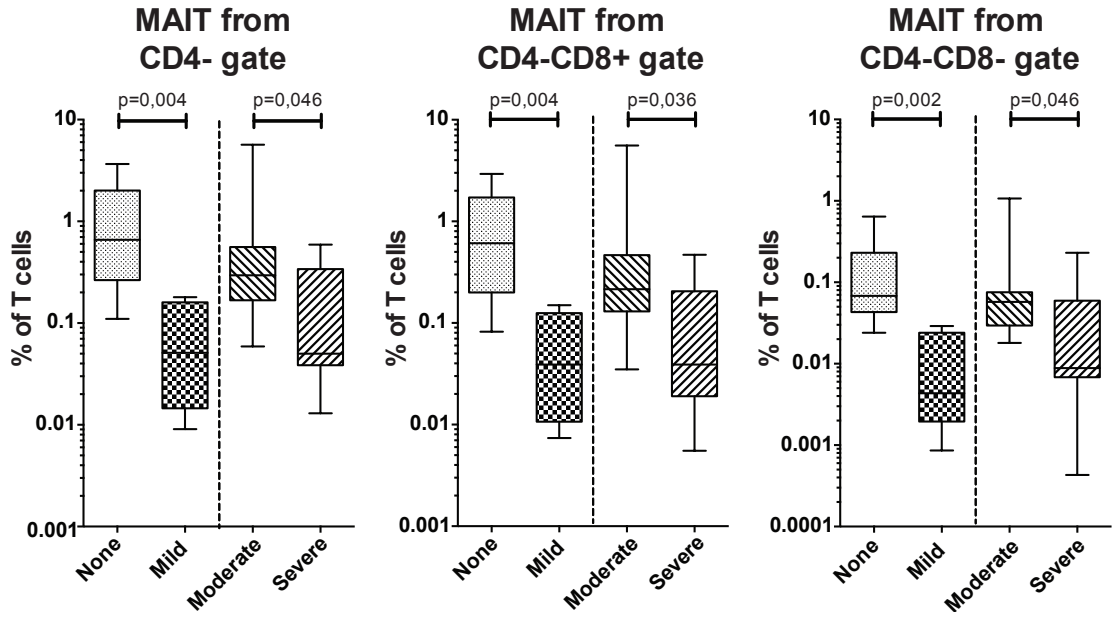

Figure 15. MAIT cell frequencies gated on CD4-, CD4-CD8+ and CD4-CD8- T cells.

Interestingly, we identified a role for MAIT cells in both aGVHD and cGVHD development. In **Paper I** we observed that patients who developed higher grades of aGVHD received grafts with lower frequencies of MAIT cells. We hypothesised that the MAIT cells in the grafts might have a protective function in aGVHD development. In **Paper II**, looking at cGVHD we observed the same pattern; lower frequencies of MAIT cells in the blood of patients with higher cGVHD. As we assessed MAIT cell frequencies at the time of cGVHD, we do not know the MAIT cell frequencies in patient blood before cGVHD development. We can thus only use MAIT cells frequencies in cGVHD as a diagnostic tool and not as a predictive tool. However, it seems that for both aGVHD and cGVHD, receiving a graft with high MAIT cell frequency or having a high MAIT cell frequency in the blood is associated to a better GVHD outcome.

Lastly, we observed an increased frequency of CD38+ cytotoxic T cells in patients with mild cGVHD compared to patients without cGVHD. No difference was observed between patients with moderate and severe cGVHD. Even though this finding is interesting, it is not entirely unexpected. CD38 expression has been linked to aGVHD development before (though we did not observe this in **Paper I**).[273] While not linked to cytotoxic T cells in cGVHD development, CD38 has been linked to cGVHD in the context of B cells. $CD38^{hi}$ plasmablasts were linked to cGVHD in a recent study.[265] We did not detect differences in

CD38+ B cell frequencies in our study. However, as we did not include CD24 we could only look at total B cells and not specifically at plasmablasts.

While the presented results are interesting, they did not identify novel subsets that may explain the pathophysiology of cGVHD in more detail. Hence, we decided to perform mass cytometry. One of the major benefits of mass cytometry is that it uses metal isotopes instead of fluorochromes to analyse markers on individual cells. This means that there is no overlap in signal, making it possible to analyse a large number of markers simultaneously on the same cell, potentially up to 100 markers. The only limiting factor is the availability of metal isotopes. This is why mass cytometry has been used more and more to immune phenotype individuals for various reasons. For instance, research has been done to assess immune variation in healthy individuals, to identify phenotype shifts in VZV infection, to identify rare immune cell subsets, to map the feto-maternal immune system, and to analyse immune phenotype after solid organ (liver) transplantation.[274-278]

In this study, we analysed 33 markers on each individual cell. Since 33 markers result in an incredible large number of possible two-dimensional plots, it is not feasible to gate all populations manually. Hence, Citrus, an automated cell clustering software was used.[279]

We performed mass cytometry on 40 patients. Due to sample limitations, only 40 of the 53 patients of the first patient cohort were analysed. Several populations, or clusters of interest, were identified. Most differences were observed between patients without cGVHD and mild cGVHD and fewer differences between patients with moderate and severe cGVHD. I will focus on two of the six clusters identified in **Paper II** to differ between patients without cGVHD and with mild cGVHD; and on both clusters identified to be different between patients with moderate and severe cGVHD. An overview of the four clusters and the cellular markers expressed by each cluster is shown in Table 4.

Table 4. Four cellular clusters identified after mass cytometry and their expression of cellular markers

	without cGVHD vs. mild cGVHD	**moderate vs. severe cGVHD**
B cells	cluster 399970 CD19+ CD39+ CXCR5+ HLA-DR+ CD38+ Ki-67+	cluster 399948 CD19+ CD39+ CXCR5+ HLA-DR+
(NK) T cells	cluster 399954 CD3+ CD57+ GzB+ $CD8^{low}$ $PD\text{-}1^{low}$ CCR4+	cluster 399981 CD3+ CD57+ GzB+ $CD8^{low}$ $PD\text{-}1^{low}$

The four clusters could be confirmed in smaller flow cytometry panels in patients from the second cohort. For this confirmation, we picked 9 of the 33 markers that we deemed to be most indicative of the cluster of interest. Nine markers were the maximum number we could analyse by flow cytometry. Moreover, we wanted to analyse as many dimensions as possible to try to mimic the high dimensional analysis of mass cytometry. We used Boolean gating to identify the clusters. Boolean gating entails that you gate for all markers on a major cell subset. After you are satisfied with the gates, the software then calculates the frequency of cells that would fit in all of the gates you tell it to incorporate in the calculation. This avoids any user bias you might encounter in conventional sequential gating. In sequential gating, you gate for one subset, display the subset and then gate for the next subset, etc. The user can adjust the gates as they see fit, which can make the subsets more accurate but, especially if the user is not blinded to the data, bias may occur. Hence,

we decided to start our flow cytometric analysis using Boolean gating and also perform sequential gating where needed.

Patients without cGVHD and patients with mild cGVHD were different in the abundancy of cluster 399954, a (NK)T cell subset and 399970, a B cell subset (Figure 16). Cluster 399954 was found at a lower abundancy and cluster 399970 in a higher abundancy in patients without cGVHD. Cluster 39954 was thought to be either an activated cytotoxic T cell or an NKT cell subset. The cells in this cluster expressed CD3, CD57, CCR4, granzyme B and to a lower extent CD8 and PD-1 (Figure 16A). As we did not include any specific NKT cell marker in the mass cytometry, nor did we have space to do so in the confirmatory flow panel, we cannot state for sure whether these cells are NKT cells or activated cytotoxic T cells. Cytotoxic T cells can also express CD57 and upon activation reduce expression of CD8.[280, 281] Since we previously identified differences between patients without cGVHD and mild cGVHD in CD38-expressing cytotoxic T cell frequencies in the first cohort, we hypothesise that it is likely that the cells in cluster 399954 are also activated cytotoxic T cells.

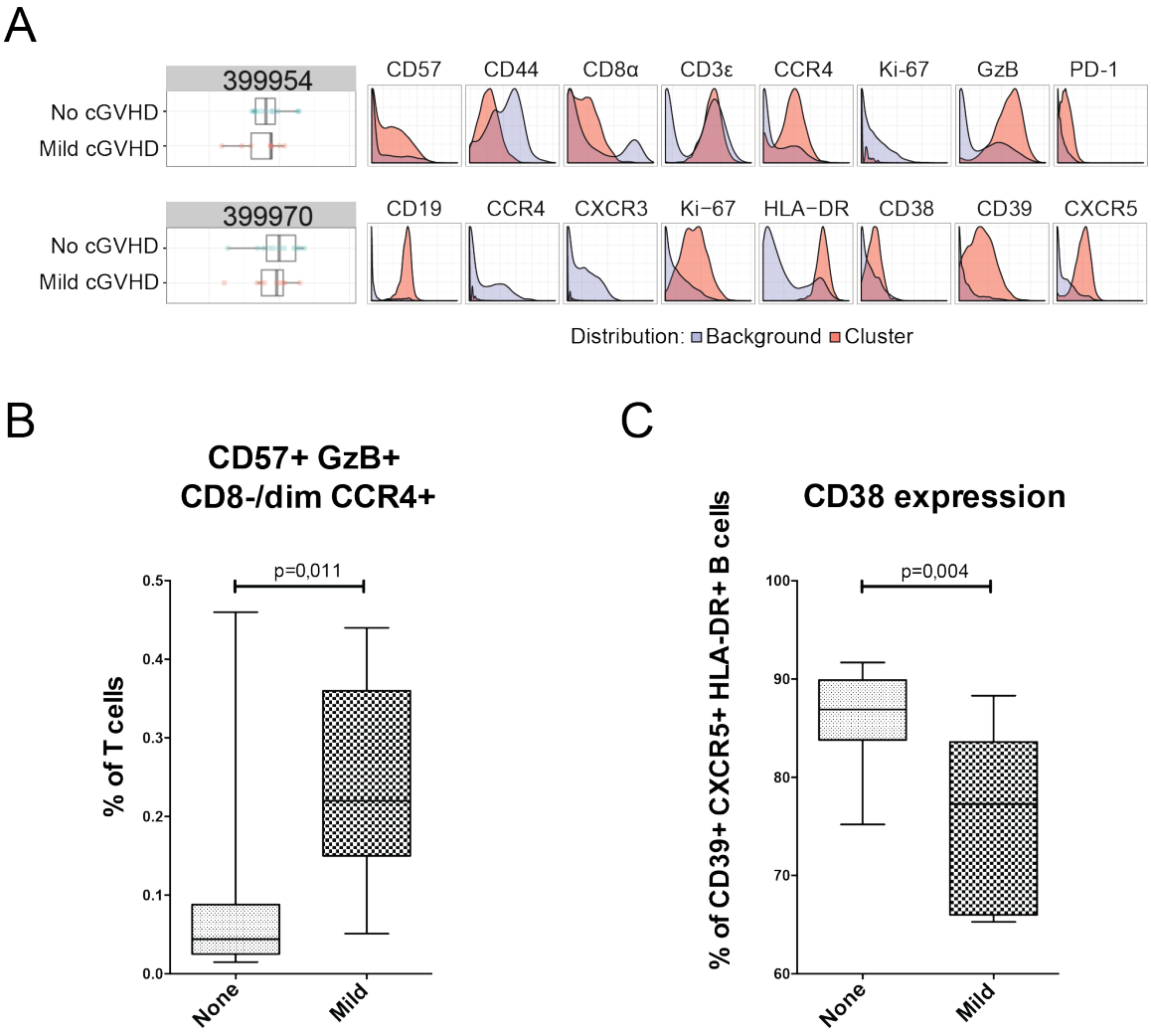

Figure 16. Mass cytometry and confirmatory flow cytometry results of two clusters that varied significantly between patients without cGVHD and patients with mild cGVHD.

In the confirmatory flow cytometry, we identified cluster 399954 by Boolean gating for a positive expression of CD3, CD57, granzyme B and CCR4, and a dim to negative expression of CD8 (Figure 16B). Similar to the mass cytometry results, patients with mild cGVHD had a higher frequency of these cells than patients without cGVHD. This indicates a more cytotoxic T cell phenotype in patients with mild cGVHD.

Unfortunately, cluster 399954 did not express CD38, hence the CD38+ cytotoxic T cells we identified, as discussed before, are not the same cells as those in cluster 399954. These are two distinct cellular subsets that differ between patients without cGVHD and mild cGVHD. However, both findings point towards a general activation of the cytotoxic T cells in patients with mild cGVHD.

The second cluster (399970) was considered to be a B cell subset, as it was positive for CD19. Moreover, the cells in this cluster also expressed CD38, CD39, CXCR5, HLA-DR and Ki-67 (Figure 16A). This would suggest an activated B cell subset undergoing proliferation. Unfortunately, we encountered problems with the Ki-67 staining in the confirmatory flow cytometry panel. We speculate this was due to using a too mild intracellular staining protocol. For fear of destroying epitopes on the cell surface, we may have used a too mild detergent, making it impossible for the Ki-67 antibody to enter the nucleus. Though we could still identify the cluster in flow cytometry by Boolean gating for CD19, CD38, CD39, CXCR5 and HLA-DR, we could not detect differences in this population between patients without cGVHD and patients with mild cGVHD. Hence, we also performed conventional sequential gating for flow cytometry.

We identified a difference in populations between the two patient groups when analysing CD38 expression on CD39+ CXCR5+ HLA-DR+ B cells (Figure 16C). This subset was found at a higher frequency in patients without cGVHD, comparable to the mass cytometry results for cluster 399970 (Figure 16A). This finding is in contrast to findings of a recent study where they correlated high frequencies of CD38hi plasmablasts with ongoing cGVHD.[265] However, that study incorporated patients with varying grades of cGVHD with most having severe cGVHD. Our cohort included only patients with mild cGVHD or no cGVHD. Additionally, since we did not include CD24 in our analysis, it is not possible to say whether the subset we identified constituted of plasmablasts. We conclude that the subset we identified is a novel subset that may be worthy of further research.

Looking at the markers expressed, we hypothesise that these are recently activated B cells that might have some form of regulatory function. Even though CD39 has not been linked to B cell regulatory function before, it has been linked to T cell regulatory function and might have a similar function in B cells.[282, 283] It would be interesting to sort these cells by flow cytometry and culture them to see whether they have any regulatory capacity *in vitro*. This was not possible within the scope of this study, but might be possible in a follow-up study with a new cohort of patients.

We also performed mass cytometry on samples from patients with moderate and severe cGVHD. These patient groups were found to differ in the abundancy of cluster 399948, a B cell subset and 399981, another (NK) T cell subset (Figure 17A). Both clusters were more abundant in patients with severe cGVHD. Strikingly, though not exactly the same, both clusters resembled the previously described clusters in patients without cGVHD and mild cGVHD (Figure 16).

The clusters were identifiable via Boolean gating but were not different between patients with moderate and severe cGVHD. Due to low sample availability at the time the

confirmatory flow cytometry was performed, we expect that the lack of statistical significance is mostly due to low power. However, as mentioned before, the effect of high immunosuppressant drugs given for long periods to these patients may also explain the lack of differences between these groups.

I will discuss the clusters one by one. Cluster 399948 (Figure 17A), resembled cluster 399970 (Figure 16A). The B cells in cluster 399948 also expressed CD39, CXCR5 and HLA-DR, but not CD38. Another difference between these clusters is that cluster 399948 was expressed at a higher frequency in more severe cGVHD, while cluster 399970 was expressed at a higher frequency in patients without cGVHD. These two B cell subsets are distinct from each other but since they co-express quite a few markers it would be interesting to further analyse them side by side. As these subsets are not well studied, it is hard to speculate how they might function in cGVHD development.

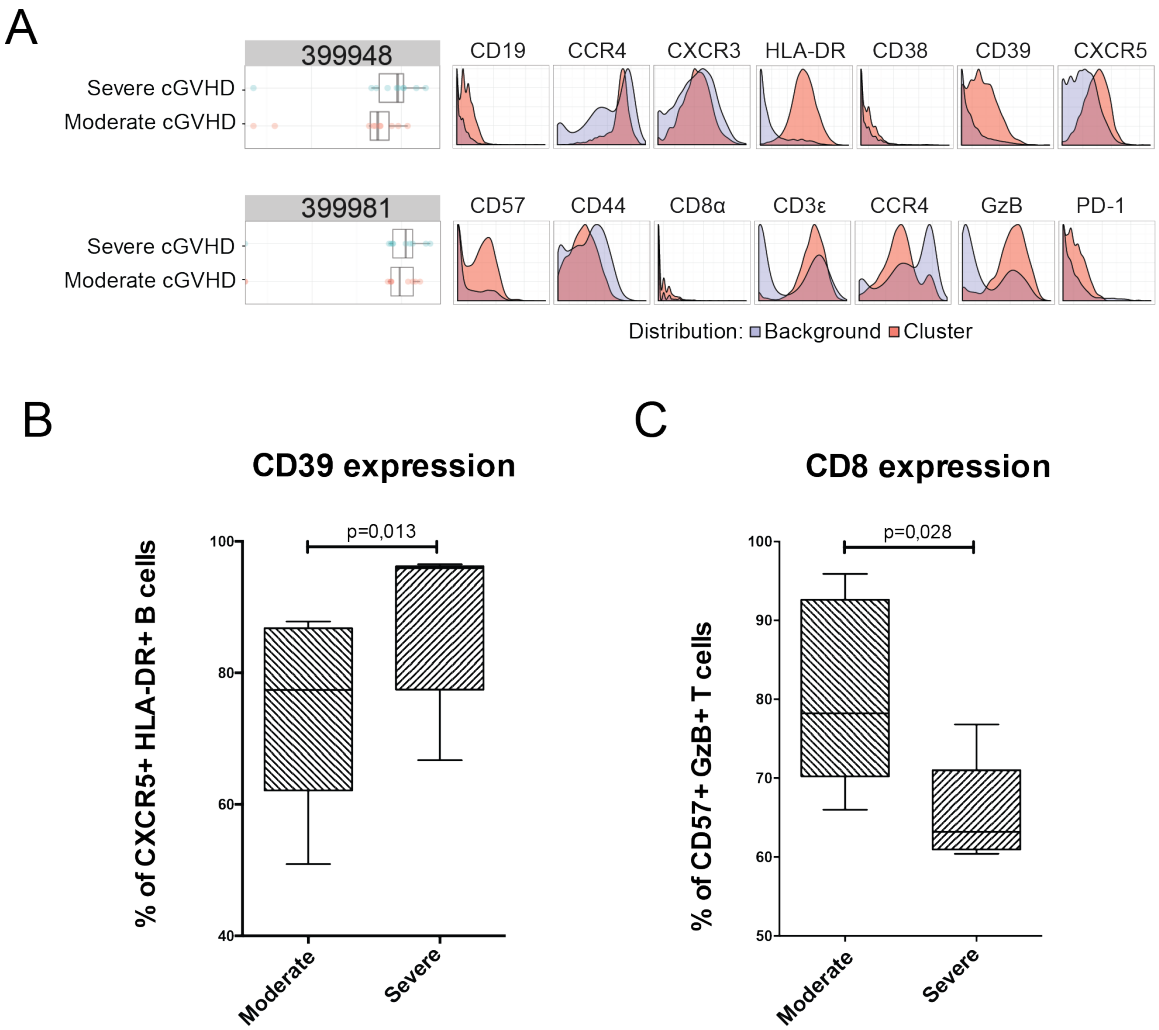

Figure 17. Mass cytometry and confirmatory flow cytometry results of two clusters that varied significantly between patients with moderate and severe cGVHD.

The second cluster that differed between moderate and severe cGVHD patients by mass cytometry was cluster 399981 (Figure 17A). This cluster resembled cluster 399954 (Figure 16A). Cluster 399981 expressed CD3, CD57, granzyme B and, to a lesser extent, CD8 and PD-1. When performing the sequential gating for this cluster in the confirmatory flow cytometry, we identified a reversed significant difference between the groups. In the mass cytometry, this cluster was found in higher abundancies in severe cGVHD patients. In the confirmatory flow cytometry though, we identified a reduced frequency of CD8 expressing

Granzyme B+ CD57+ T cells in patients with severe cGVHD (Figure 17C). This difference in mass cytometry and flow cytometry results could be due to sample size or it could be due to the fact that in the mass cytometry 33 markers contributed to the identification of the cluster, while in the flow cytometry, the population was identified by just 4 markers. The population identified by mass cytometry may be a smaller subset of the population identified by flow cytometry. As such the frequencies may be inverted. Interpreting the results of the flow and mass cytometry is difficult. While it may seem contradictory to have less cytotoxic T cells in blood in patients with more severe cGVHD, we speculate that the cytotoxic T cells might have migrated towards the inflamed tissue, similarly to the MAIT cells, as discussed before. This is something that should be explored by analysing biopsies from patients. Until then, we can only speculate on whether this finding is truly something involved in the pathophysiology of cGVHD or simply a bystander effect of the disease.

The mass cytometry findings could, to a large extent, be confirmed in smaller flow cytometry panels. Due to sample availability, the confirmatory flow cytometry panels may have been underpowered and may explain why we could not confirm all clusters or only confirm them by sequential gating. Most importantly we feel that it is possible to use multi-dimensional methods to identify novel subsets, which may then be converted and used in more routine settings.

There is one important aspect of cGVHD that we have not discussed in **Paper II**. In the paper, we compared patients with moderate to severe cGVHD. However, all moderate cGVHD patients and all severe cGVHD patients are not alike. As cGVHD can manifest in many different organs, these patients could also be split by cGVHD organ involvement instead of total severity. For instance, compare patients with lung-associated cGVHD to patients without lung-associated cGVHD. This type of analysis was not performed in the paper, however, it is something that we are currently working on. As we are interested in diagnostic markers, the mass and flow cytometry results could help identify markers that would help identify affected organs.

To illustrate, preliminary findings indicate a reduced frequency of MAIT cells in the blood of patients with GI tract-affected cGVHD regardless of moderate or severe cGVHD classification (Figure 18). This is in line with the hypothesis that MAIT cells migrate towards the affected GI tract.

Additionally, before mass cytometry was performed, half the sample was stimulated with PMA and ionomycin. Hence, we can also analyse the differences between patient groups when their lymphocytes are activated in terms of cellular markers and cytokine production. We are currently analysing these results.

In this study, we aimed to identify potential diagnostic markers for cGVHD and gain insights into the pathophysiology of cGVHD. Since we did not analyse our results in a completely independent second cohort, we cannot say with certainty that the markers identified are truly

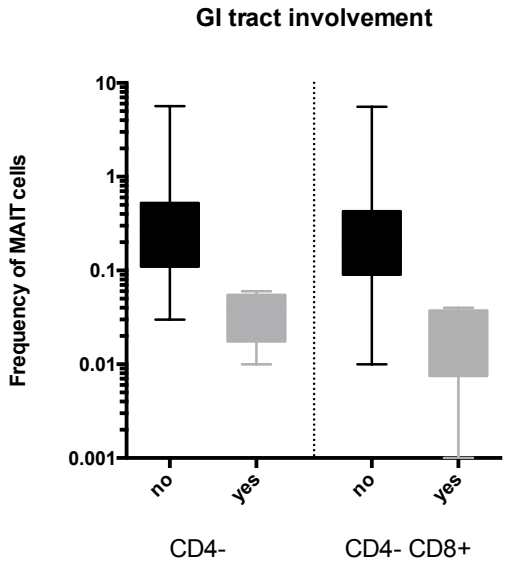

Figure 18. MAIT cell frequencies gated on CD4- and CD4-CD8+ T cells, comparing moderate and severe cGVHD patients on GI tract involvement.

biomarkers. As described in detail in the 2014 report of the NIH cGVHD biomarker working group, biomarkers need to be confirmed in at least two independent cohorts.[284] Hence, our results need to be validated in another cohort before they can be considered biomarkers.

However, they do shed some light on the pathophysiology of cGVHD. For instance, there appears to be a role for MAIT cells in both aGVHD and cGVHD. Though they are a small subset in blood, frequency variations appear to have some predictive value in aGVHD and diagnostic value in cGVHD. Thus, we conclude that MAIT cells may have a function in GVHD development. One suggested function is migration towards inflamed tissue to exert an effector function there. It is unclear whether they have a dampening or a pro-inflammatory effect. More research into this is needed. One first step would be to analyse biopsies, preferably both inflamed and non-inflamed parts of tissue from the same patient. This would indicate whether MAIT cells are recruited to inflamed tissue or not.

Perhaps the most intriguing aspect of the cGVHD study is the possibility to use multidimensional methods to discover novel subsets. In this study, the mass cytometry results could be interpreted and translated into smaller flow cytometry panels. Unfortunately, we could not identify all of the same differences in these smaller flow cytometry panels. We feel that small sample size could be a factor in the lack of statistical significance.

Similar as for **Paper I** on aGVHD, we feel that the results are interesting and worthy of further research. As such, several future steps should be taken to further elucidate and confirm these findings. We will discuss some of these in the "concluding remarks and future aspects" section.

To conclude, this part of the discussion has focused on two complications where graft and recipient co-existence does not work as intended. A war has broken out between the graft and the recipient. They are two different types of war. Acute GVHD could be seen as a fast and overwhelming invasion and destructive war. Chronic GVHD on the other hand is slower, stays under the radar for longer and is more of a long-lasting, exhaustive occupation type of war. Fortunately, there are also a lot of patients where these severe grades of aGVHD and cGVHD do not occur and who do well post-HSCT. The fact that some patients do not suffer from these severe diseases is one of the main reasons why HSCTs are still performed and why so much research is put into elucidating the mechanism behind the complications. A better understanding will hopefully improve the HSCT treatment, resulting in more and more patients who do not need to suffer from GVHD. Compared to HSCT outcomes of just a decade ago, globally, HSCTs have benefited from the efforts of researchers around the world, as severe grades of GVHD are slowly decreasing in almost all centres.

4.2 MIXED CHIMERISM: CO-EXISTENCE TO THE EXTREME

In broad terms, when we talk about the co-existence of the donor graft-derived immune cells and the recipient cells, we refer to the immune tolerance of the graft to the recipient. However, post-HSCT, in MC there is not only a tolerance of the donor-derived hematopoietic system towards the patient tissues, there is also a tolerance of the patient's own recipient hematopoietic system towards the donor's. This is a quite extraordinary phenomenon that most often only occurs early post-HSCT. Usually the donor-derived hematopoietic system triumphs in the end, be it on its own or helped by DLIs. However, there are special cases where long-term stable MC occurs and persists. These patients are still mixed chimeras even several years post-HSCT.

Long-term stable MC has not been studied to a great extent or detail. At our centre we were fortunate to have an extensive patient database and practice of regularly performing chimerism analyses on all transplanted patients. Hence, we could identify a cohort of living patients displaying stable MC more than 5 years post-HSCT.

One major limitation of the study is that we only included patients still alive at least 5 years post-HSCT. It is possible that we introduced a selection bias by recruiting patients in this way. The only option to avoid this is to include patients from the time of HSCT and following them prospectively. This was not possible for the scope of this PhD programme.

Since not much is known about these rare patients with long-term stable MC we decided to analyse them further to see if we could elucidate the development of MC. Our specific aim for the study, as presented in **Paper III** and **IV**, was to gain insight into the mechanism behind long-term stable MC in patients that underwent HSCT to treat non-malignant disorders. Our patient cohort with MC was compared to the control group; a cohort matched for age, disorder, gender and time post-HSCT who displayed stable DC. We attempted to elucidate the immune phenotype and function of the hematopoietic systems in these two patient cohorts. We aimed to answer questions such as whether MC affects the patients' quality of life and whether both hematopoietic systems were functional or not.

The study started by comparing the two patient groups for clinical parameters to identify whether we could find any clinical factors associated with MC development. As discussed in detail in **Paper III**, most clinical characteristics were similar for the two patient groups. We identified a significant difference in donor type, i.e., HLA-identical sibling or HLA-(mis)matched unrelated, between the patient groups (p=.005). Patients who developed MC were more likely to have an HLA-matched sibling donor than patients with DC. Moreover, patients with MC received grafts from younger donors (p=.017), a phenomenon also previously described in the literature.[285] However, as donor age and donor type are likely to be correlated, we performed a multivariate statistical analysis. This demonstrated that donor type was more associated with MC development than donor age.

Additionally, patients with DC were more prone to develop aGVHD than patients with MC. Grade II or more severe aGVHD has previously been identified as predictive of DC.[174, 285-288] Higher incidence of aGVHD in patients with DC could also be linked to differences in donor type between the patient groups as HLA-identical sibling donors are more likely to be matched also on minor histocompatibility antigens than matched unrelated donors. Hence, we performed a multivariate analysis on the effect of donor type and aGVHD on MC development. Again, donor type was more important for MC development than occurrence of aGVHD.

We concluded that a sibling donor was positively correlated to MC development. This is not entirely a surprising result. As mentioned, sibling donors are usually more matched on minor histocompatibility antigens than unrelated donors. As such, the new (donor) and the old (patient) hematopoietic systems are less likely to recognize each other as non-self. If they do not recognize each other as non-self, they are more likely to tolerate each other and co-exist peacefully.

This finding in itself can be considered promising news for the solid organ transplantation field. As mentioned in the introduction section, that field is highly interested in MC. Quite a few studies have attempted to develop a transient MC in a patient before an organ is transplanted.[178-181] The idea is to induce tolerance in the patients hematopoietic system to the donor organ during the period of MC. After some time the donor hematopoietic system will be rejected and the hope is that the patient's immune system will no longer recognize the transplanted organ as foreign. The patients would not need to be on immunosuppressive drugs for the rest of their lives. This procedure would primarily be an option for living donor solid organ transplantations especially if MC needs to be developed days or weeks prior to the organ transplantation. Living donor organ transplantations often use solid organs from a first degree relative. As such, identifying sibling donors as playing a major role in MC development could be interesting for the solid organ transplantation field.

Interestingly, we also identified a potential role for HLA-C in MC development. HLA-C mismatches have been associated with poor HSCT outcome.[289, 290] However, in a recent study in our centre, no adverse effect on HSCT outcome was observed for patients transplanted with HLA-C mismatched grafts.[291] In **Paper IV** we observed that patients with DC received grafts with more HLA-C mismatches than patients with MC. As HLA-C mismatches are more likely to occur in unrelated donors than in HLA-identical sibling donors, it is yet again a sign for the important role of sibling donors in MC development.

HLA-B18 was also identified as having a putative role in the development of MC. None of the patients with MC had an HLA-B18 allele, while almost a third of the patients with DC did (p=.039; **Paper III**). The reason for this is hard to pin down. As far as we are aware this HLA-allele has not been linked to chimerism or any other outcome post-HSCT. It has been linked to other disorders, such as lymphoproliferative disease after solid organ transplantation[292], skin squamous cell carcinoma after renal transplantation[293], and type 1 autoimmune hepatitis[294]. However, the link to chimerism post-HSCT is not entirely clear. It could be due to chance as the patient groups were quite small. Further research in larger patient groups could potentially clarify this.

The two patient groups were also compared for quality of life parameters, e.g., rate of infections, ability to work, etc. Strikingly, patients with MC were equally well or arguably better off than patients with DC for several parameters after HSCT. For instance, immunisation response to vaccinations was similar between the two patient groups (**Paper IV**). Also, none of the patients with MC developed blood stream infections (BSIs) post-HSCT, while this occurred in almost a third of the patients with DC (p=.039; **Paper III**). However, the study design did not allow us to elucidate whether the BSIs were due to DC development or vice versa. Moreover, more than 5 years post-HSCT, the two patient groups were similar in terms of infection rate, medication usage, ability to work or study fulltime, sick leave and other quality of life parameters (**Paper IV**).

We conclude that while there may be some differences between patients with MC and DC in terms of infections early post-HSCT, the patient groups are quite similar in the long-term. Patients with MC do not seem to suffer unduly from being mixed chimeras.

Despite similar quality of life post-HSCT, MC is seen as detrimental in some centres. As such it is often treated with DLIs to force the donor hematopoietic system to take over. However, DLIs are not without risk, being linked to GVHD development in most studies, especially when given in bulk dose rather than an escalating dose.[152, 295-297] Since aGVHD is not desirable, especially in patients with non-malignant disorders, we argue that DLIs should not be given immediately after MC is observed. Instead, we suggest patient monitoring to make sure the MC stabilizes. If it does not stabilize, it may indeed indicate graft rejection, especially shortly post-HSCT, and then DLIs should be given. However, if the MC stabilizes and/or if the patient is many years post-HSCT without any adverse symptoms, perhaps nothing should be done. If a long-term stable MC develops it may not necessarily be a bad thing. These patients are likely to develop less aGVHD, less blood stream infections and are in good shape in the long term.

To understand MC better, we decided to immune phenotype them extensively. Similarly, as for the studies described in **Paper I** and **Paper II**, we looked at soluble and cellular characteristics of the patients at time of inclusion in the study (**Paper IV**).

Patients with MC had lower plasma levels of IL-4, IL-12 (p40) and G-CSF. One of the most straightforward explanations could be that patients with MC have a higher cell turnover and thus a higher cytokine consumption. For instance, this could be possible in the case of infections. However, as the patient groups did not differ in infection rate or other cytokine levels, this seems unlikely. Another explanation could be that patients with MC have a slightly lower inflammatory environment than patients with DC. This seems likely as IL-12 (p40) is a classic pro-inflammatory cytokine and reduced levels would indicate a less inflammatory environment. Though the patient groups had similar infection rates at time of inclusion, patients with DC did suffer more inflammatory conditions early post-HSCT than patients with MC, e.g. more aGVHD and blood stream infections.

While it is hard to say exactly why these three cytokines were lowered in patients with MC, it is interesting to see how they correlate with other findings in the patients with MC. For instance, G-CSF is known to suppress platelet function[184-186, 298], and as such we expect patients with MC to have more platelets than patients with DC. Indeed, patients with MC had increased platelet counts while white blood cell and neutrophil counts were similar. As this difference in platelet count was small (MC 239.000 platelet/mL vs. DC 198.000 platelets/mL), it is unlikely to hold true clinical value. It is difficult to see how this small difference could impact the patient.

Plasma levels of IL-4 were also lower in patients with MC. IL-4 is known to stimulate Th2 differentiation.[299, 300] Hence, patients with MC with a lowered IL-4 level should be more skewed towards a Th1 phenotype as compared to patients with DC. One of the main cytokines produced by Th1 cells, IFNγ, has been shown to promote B cells to class switch towards IgG3. Moreover, the same study demonstrated IL-4 directly inhibited expression of IgG3.[301] As such we would expect increased levels of IgG3 and IFNγ in patients with MC. Even though IFNγ levels were similar between the patient groups, we did observe an increased level of IgG3 in patients with MC (Figure 19).

To the best of our knowledge, IgG and specifically IgG3 levels have not previously been linked to DC. It has been negatively associated to donor-specific HLA antibodies and AB0 mismatching in (solid organ) transplantation[302-304], though there was no correlation between high IgG3 and AB0 mismatches in the patients with MC in this study. Patients with an above median IgG3 level were AB0 matched, had high Karnofsky performance status and did not have any indications of autoimmune diseases. Hence, the IgG3 increase does not

seem to be related to an allo-mediated response. It could simply be due to the observed lowered levels of IL-4. Even though IgG3 is negatively linked to solid organ transplantation outcomes, other studies identified a potential protective role for IgG3. A quite recent study discussed a case report of an adult patient suffering from recurring herpes zoster virus infection due to an IgG3 deficiency.[305] Another study reported severe recurring infections in 6 children with IgG3 deficiencies.[306] Though these cases are not comparable to the situation post-HSCT in this study, it is worth noting again that the patients with DC with lower IgG3 levels suffered from more infections early post-HSCT than the patients with MC did. Even though the infection rate was similar at time of inclusion in the study, we can speculate that perhaps patients with MC were still slightly more protected against infections than patients with DC due to increased IgG3 levels.

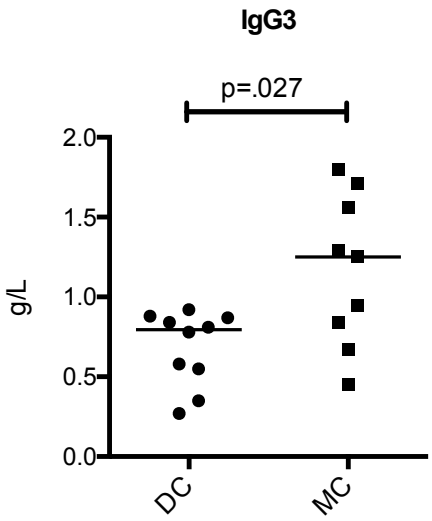

Figure 19. IgG3 levels in patients with full donor chimerism (DC) and mixed chimerism (MC).

Interestingly, IgG3 was also positively correlated to platelet counts in patients with MC. Hence, platelet counts, and with it G-CSF levels, are linked to IgG3 and IL-4. In our experimental set-up it is impossible to state if the increased levels of IgG3 were due to the lowered levels of IL-4 or due to another reason. At the moment, we do not know which phenomenon came first, IgG3, platelets or IL-4. To be sure, longitudinal follow-up and immune phenotyping is needed.

Unfortunately, the reduced levels of IL-12 (p40) may complicate matters a bit more than I first let on. As stated, IL-12 (p40) is a pro-inflammatory cytokine and lower IL-12 (p40) levels thus fit the less inflammatory environment hypothesis of patients with MC. Moreover, IL-12 (p40) is also known to stimulate cells to produce IFNγ.[307] As discussed previously, IFNγ can induce B cells to switch to IgG3. However, IL-12 (p40) can also promote Th1 cell differentiation and is important for NK cell function.[308, 309] Hence, patients with MC should be less skewed towards a Th1 phenotype. This is in contrast to what we speculated earlier, regarding the finding of low IL-4 levels skewing these patients towards a more Th1 phenotype. Moreover, NK cell frequencies were similar between the patient groups in contrast to what we might have expected due to the difference in IL-12 (p40) levels. The only thing close to an NK cell phenotype difference was an increase in CD56+ CD8+ and CD94+ CD8+ T cells in patients with MC. This subset is either an NKT cell subset or an activated CD8+ T cell subset. Since no additional markers were added to specify further, we unfortunately cannot say for sure. As mentioned in the cGVHD study, CD8+ T cells can express CD56/CD57 and not be classified as an NKT cell.[280, 281, 310] Additional markers are required for confirmation.

Cytokines are part of an intricate and complex process. They influence each other making it difficult to truly understand the impact of single cytokine levels as a whole. No single cytokine is the most important. Hence, while it seems likely that patients with MC have a slightly less inflammatory environment than patients with DC, it is hard to say this for sure as some results can be interpreted in conflicting ways. As mentioned, a longitudinal follow-up and larger cohorts are needed.

The patient groups were also compared for cellular immune phenotype and were found to be quite similar. The only differences observed between the groups were platelet counts and the increased frequency in CD56+ CD8+ and CD94+ CD8+ T cells, as mentioned in the previous paragraphs. The major cellular subsets, such as T cells, B cells and NK cells were the same. The radar graphs in Figure 20 demonstrate the similarity for these cellular subsets in the patient groups.

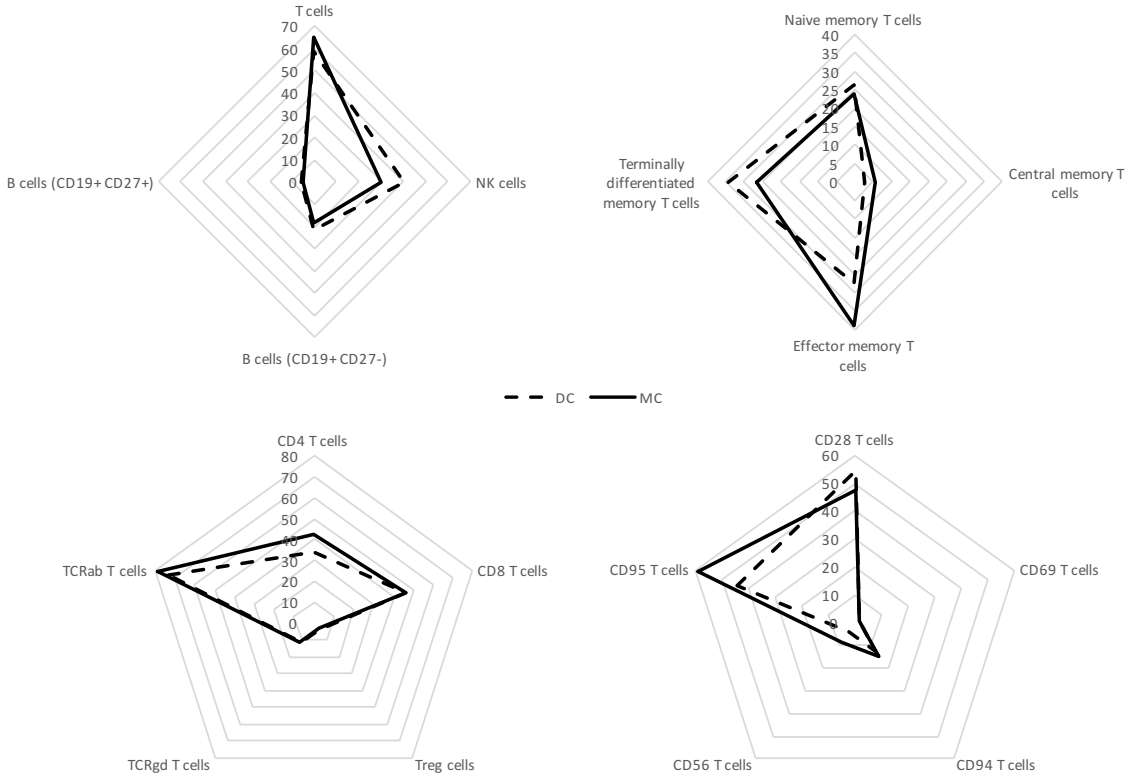

Figure 20. Cellular characterisation of mixed chimerism (MC) and full donor chimerism (DC) patients.

We also looked at the protein expression of certain key proteins in the T cell activation cascade by western blot. As we could not separate the cells prior to analysis, the western blot was performed on whole lymphocyte populations. Two of the proteins analysed showed interesting results, ZAP-70 and LCK. The function of both proteins in the T cell activation cascade has been discussed in the relevant introduction section. ZAP-70 was found at lower levels in patients with MC compared to patients with DC. LCK levels were similar between the patient groups. Both proteins are vital in the activation cascade. A reduced expression of ZAP-70 or LCK has been linked to T cell anergy.[21, 311, 312] As such, it can be argued that the reduced levels of ZAP-70 in patients with MC may dampen T cell activation, as they have a reduced ability of TCR signalling. This may affect their ability to react to antigens. Since the patients are seemingly still able to react to common pathogens, perhaps the reduced signalling contributes towards the tolerance between the two immune systems. They may still respond to strong signals from pathogens. However, weaker signals from patient and donor antigens, seeing as how the vast majority of the patients with MC were HLA matched, may not be sufficient to initiate an effective TCR signalling cascade. To determine whether reduced ZAP-70 levels could drive tolerance between the systems or whether it is merely a symptom of tolerance after it has developed, one would need to find out when the reduced level of ZAP-70 emerges post-HSCT. Is this a phenomenon that occurs early post-HSCT or does it appear after a stable long-term MC has developed?

Moreover, is the ZAP-70 deficiency present in only the patient- or only the donor-derived lymphocytes, or in both? While we cannot be sure, we hypothesise that the lowered ZAP-70 expression was present in both patient- and donor-derived lymphocytes. Irrespective of whether a patient with MC had a high recipient chimerism (predominantly recipient cells) or a low recipient chimerism (predominantly donor cells), ZAP-70 expression was lowered in patients with MC. Thus, it seems likely that both donor- and recipient-derived cells had lower ZAP-70 expression in patients with MC, maybe indicating a global tolerant state of the lymphocytes.

To better understand the impact of the protein expression and as a functional indicator of the hematopoietic cells, we decided to see if we could correlate the findings to immune phenotype. While ZAP-70 expression was different between patients with MC and DC, it was not correlated to immune phenotype for either patients with MC or DC. On the other hand, LCK which was similar between the groups, was correlated to immune cell phenotype of patients with MC.

LCK expression correlated to a higher frequency of effector memory cells in the CD4+ T cell and total αβ T cell compartment (Figure 21). Strikingly, this difference was not observed in CD8+ T cells. As far as we are aware, both CD8+ and CD4+ T cell activation requires LCK involvement. To complicate matters further, γδ T cells displayed the reverse correlation pattern for LCK and effector memory phenotype. Effector memory γδ T cells were correlated to less LCK expression. As γδ T cell activation and memory differentiation is still not elucidated entirely, we can only hypothesise as to the cause of the reversed LCK expression pattern in these cells. One explanation could be that these non-conventional T cells have different downstream signalling kinetics upon activation than αβ T cells, making them less dependent on LCK and ZAP-70 expression.[313, 314]Another explanation could be that the classical method of identifying effector memory T cells by CCR7 and CD45RO expression does not suit γδ T cells.

Another way to determine functionality is to stimulate lymphocytes and measure activation and cytokine production. We performed a stimulation experiment with PMA and ionomycin. As discussed in the methods section, this will stimulate lymphocytes. The cells were stimulated for four hours and then IFNγ and IL-2 production was measured. For optimal IL-2 production, slightly longer stimulation times are usually recommended but this was not possible due to sample availability.[315]

Patients with MC and DC had similar cytokine production regardless of gating on total T cells, CD4+ T cells, CD8+ T cells or memory (CD45RO+) T cells after PMA stimulation. We did see a difference in patients with MC and DC in the control experiment which was incubated for four hours without stimuli. This can be seen as the baseline cytokine production, similar to the unstimulated setting in the aGVHD study in **Paper I**. Patients with MC produced more IL-2 when unstimulated than patients with DC for all the aforementioned cellular subsets. No differences were seen in IFNγ production. The frequencies of IL-2 producing cells were quite low (1-4% depending on the subset), which is to be expected in an unstimulated control.

It is surprising that patients with MC had a higher IL-2 steady state production than patients with DC. IL-2 has been shown to promote T cells towards effector memory phenotypes.[316] As there was a correlation between effector memory phenotype and LCK in patients with MC, we hypothesised that there might also be correlation between LCK and IL-2 production. Indeed, we could detect correlations between LCK and IL-2 & IFNγ or only IFNγ producing CD4+ T cells (Figure 22). We could not detect a correlation for CD4+ T

cells only producing IL-2 nor did we find a direct correlation between cytokine production and effector memory phenotype. It is not easy to conclude why we could not correlate IL-2 only producing cells to LCK or directly to effector memory phenotype. It could be because LCK expression was measured on total lymphocytes and that the cytokine production was limited to smaller cellular subsets. Additionally, it is likely that the sample size of these experiments limit the ability to draw firm conclusions.

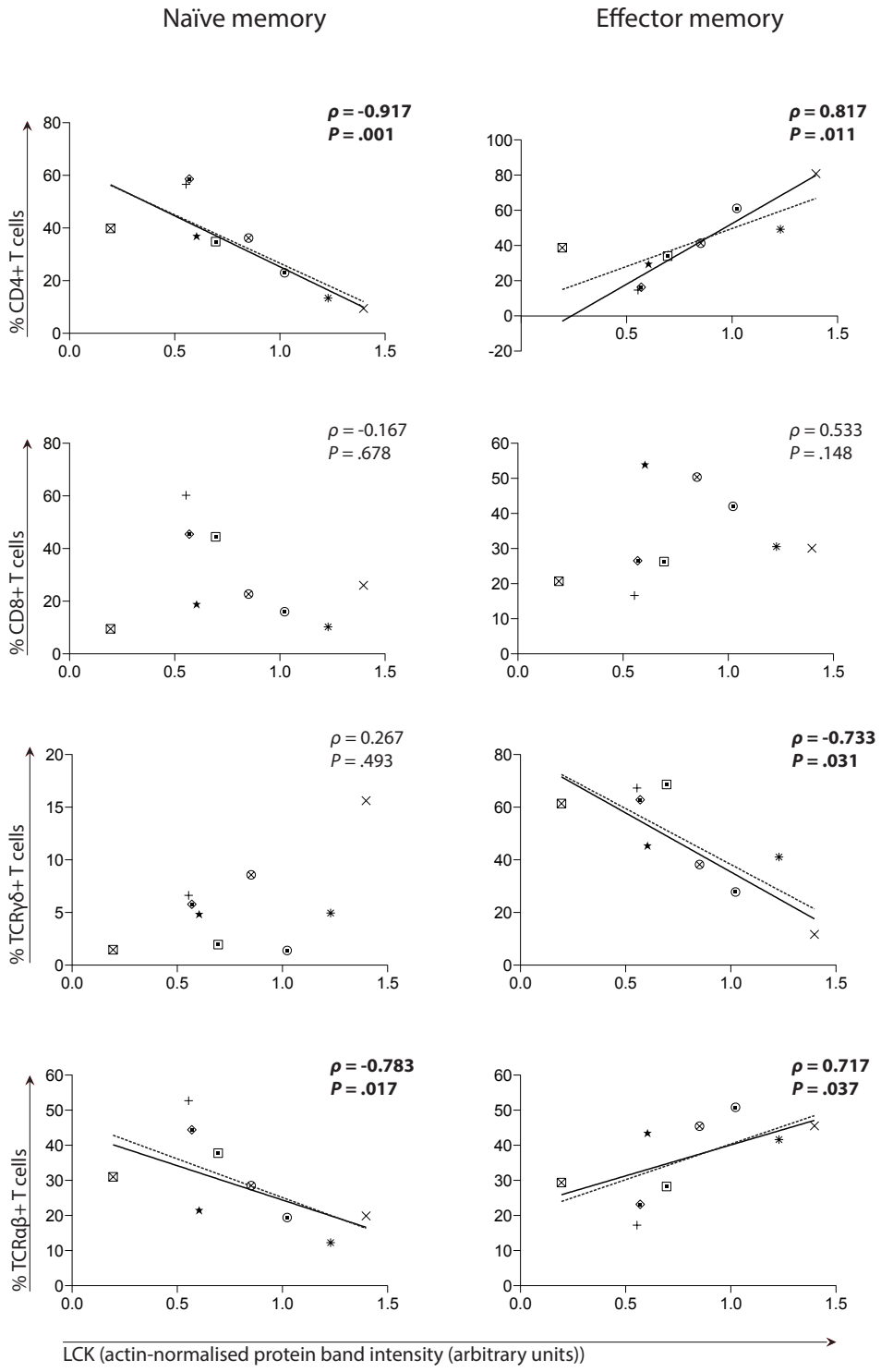

Figure 21. Memory differentiation correlated to LCK expression in mixed chimerism (MC) patients. Two different regression analysis methods were used, as there is no unified linear regression method best suited for non-normal data. The dotted line indicates the least squares fit linear regression analysis and the solid line indicates the robust fit linear regression analysis.

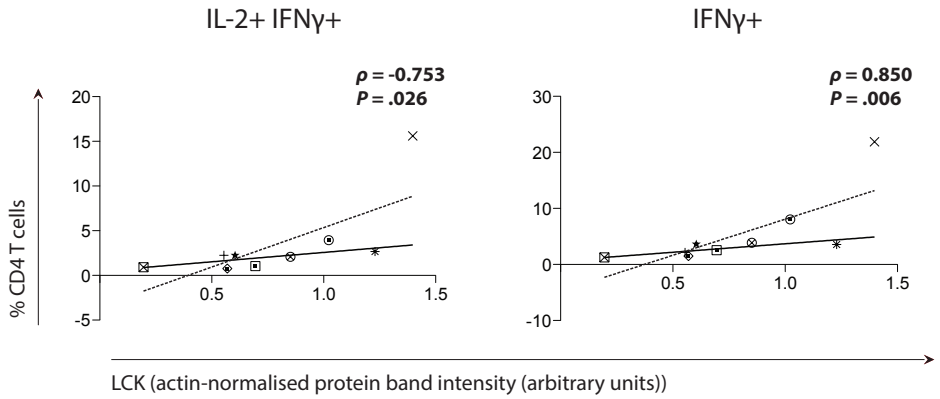

Figure 22. Cytokine-producing CD4+ T cells correlated to LCK expression in mixed chimerism (MC) patients. Two different regression analysis methods were used, as there is no unified linear regression method best suited for non-normal data. The dotted line indicates the least squares fit linear regression analysis and the solid line indicates the robust fit linear regression analysis.

To understand MC development better we looked at patients with MC in more detail. We started with investigating how patients developed MC over time. To this end, we collected chimerism analysis results performed for these patients over the years post-HSCT for T, B and myeloid cells in blood (Figure 23).

In general, frequencies of recipient-derived cells fluctuated the most during the first couple of years. To find out if these fluctuations were true fluctuations and not merely due to the effect of multiple chimerism analyses during the first years and only occasional analyses later years post-HSCT, we calculated the relative fluctuations for three different time periods post-HSCT. By dividing the sum of the fluctuations by the number of times chimerism analysis was performed during the period of interest, we could demonstrate a significant increase in fluctuations in the 0-2 years period post-HSCT as compared to the 2-3 years period post-HSCT (**Paper III**). Thus, patients became more stable in their MC status over time. We hypothesise that this is in part due to the fact that the first couple of years are turbulent. Patients have undergone extensive conditioning regimens, engrafted an entire new hematopoietic system and can become sick with aGVHD, cGVHD and infections. It is possible that as the patient undergoes such intense events, the interaction between patient- and donor-derived hematopoietic systems are affected as well. The patients with MC need time to develop a calmer, less inflammatory environment before stable MC can develop. In some patients, the fluctuations already flatten out as early as 1-2 years post-HSCT, while in others it can take up to 5 years post-HSCT.

Another interesting finding was that the fluctuations were not necessarily uniform between cellular subsets. For instance, T cell frequencies fluctuated much more than B cells. This is partly due because T cells engraft much faster than B cells. However, it could also indicate a T cell mediated role in developing tolerance. For instance, patients who developed long-term stable MC were less likely to have blood stream infections and aGVHD development post-HSCT. We could speculate that if there are less infections and no aGVHD, there are less danger signals and costimulatory signals for the donor-derived hematopoietic cells to respond to. Thus, the donor-derived hematopoietic cells are less inclined to respond to recipient-derived cells in a negative way. Moreover, as patients with MC were more likely to receive grafts from HLA-identical siblings, fewer histocompatibility mismatches make it less likely for the donor-derived hematopoietic cells to negatively react to recipient cells. The reverse is also true for the remaining recipient-derived hematopoietic cells. The lack of costimulatory signals and histocompatibility mismatches may make them less likely to react to the donor-derived hematopoietic cells. Thus, tolerance is born and with it stable MC.

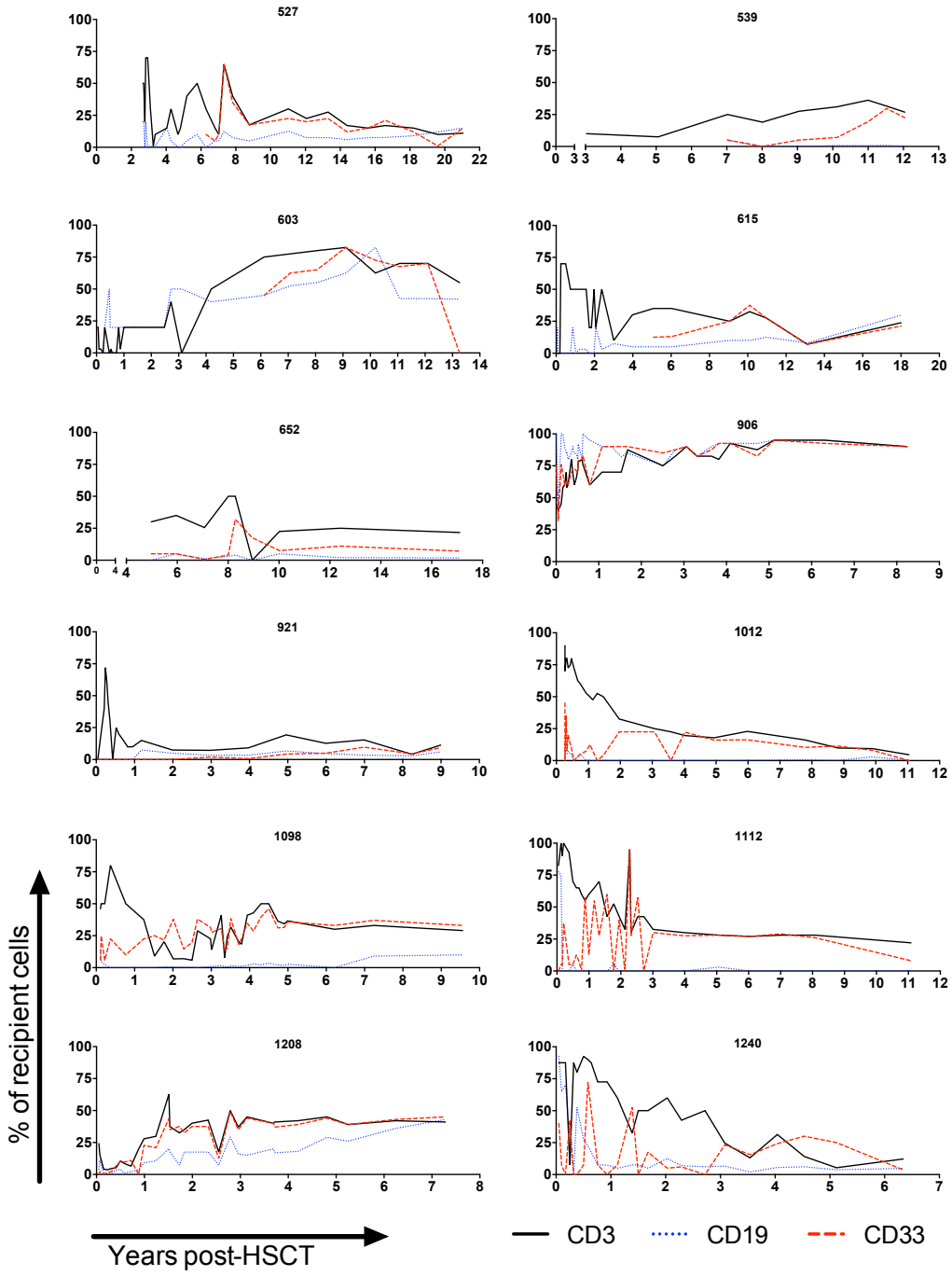

Figure 23. Chimerism pattern for 12 mixed chimerism (MC) patients post-HSCT.

These chimerism analyses indicate that in broad terms, patients with MC are chimeric for the major immune cell subsets; i.e. T, B and myeloid cells. It does not tell us whether the recipient- and donor-derived hematopoietic systems are fully present or whether they have found niches within the immune system. For instance, perhaps all γδ T cells are of recipient origin and all αβ T cells are of donor origin. To answer this, we sorted additional cellular subsets by flow cytometry and performed additional chimerism analyses. This showed that CD4+ T cells, CD8+ T cells, γδ T cells and NK cells in almost all patients with MC were of donor and recipient origin (Figure 24). In general, the frequencies of these four cell subsets followed the trend of total T cells, e.g., if a patient had a high frequency of recipient-derived T cells, the frequency of recipient-derived cells for these four other subsets was high as well. Unfortunately, due to limitations in sample availability, we could only sort

these four subsets. However, it does indicate, that on a slightly deeper level, patients with MC are still mixed. They appear to have both recipient- and donor-derived cells in all subsets.

The next question now was whether all cells were functional. Can both recipient and donor-derived cells be activated and produce cytokines? As patients with high recipient chimerism (predominantly recipient cells) were capable of fighting of pathogens, we expected that their recipient cells should retain some functionality. However, would that truly be so, and how would the recipient cells of patients with low recipient chimerism (predominantly donor cells) behave? To answer this, we performed a stimulation experiment with PMA and looked at IFNγ and IL-2 production. After stimulation, we sorted total T cells for their production of IFNγ and IL-2 (**Paper IV**) and then performed another chimerism analysis on the sorted populations. Unfortunately, we could not sort enough cells for all patients with MC. However, the patients we could analyse showed that almost all had both donor and recipient cells capable of producing cytokines (Figure 24). It is striking to see that some of the patients with low recipient chimerism (for instance, unique patient number (UPN) 527, 615 and 921) retained functionality in recipient cells.

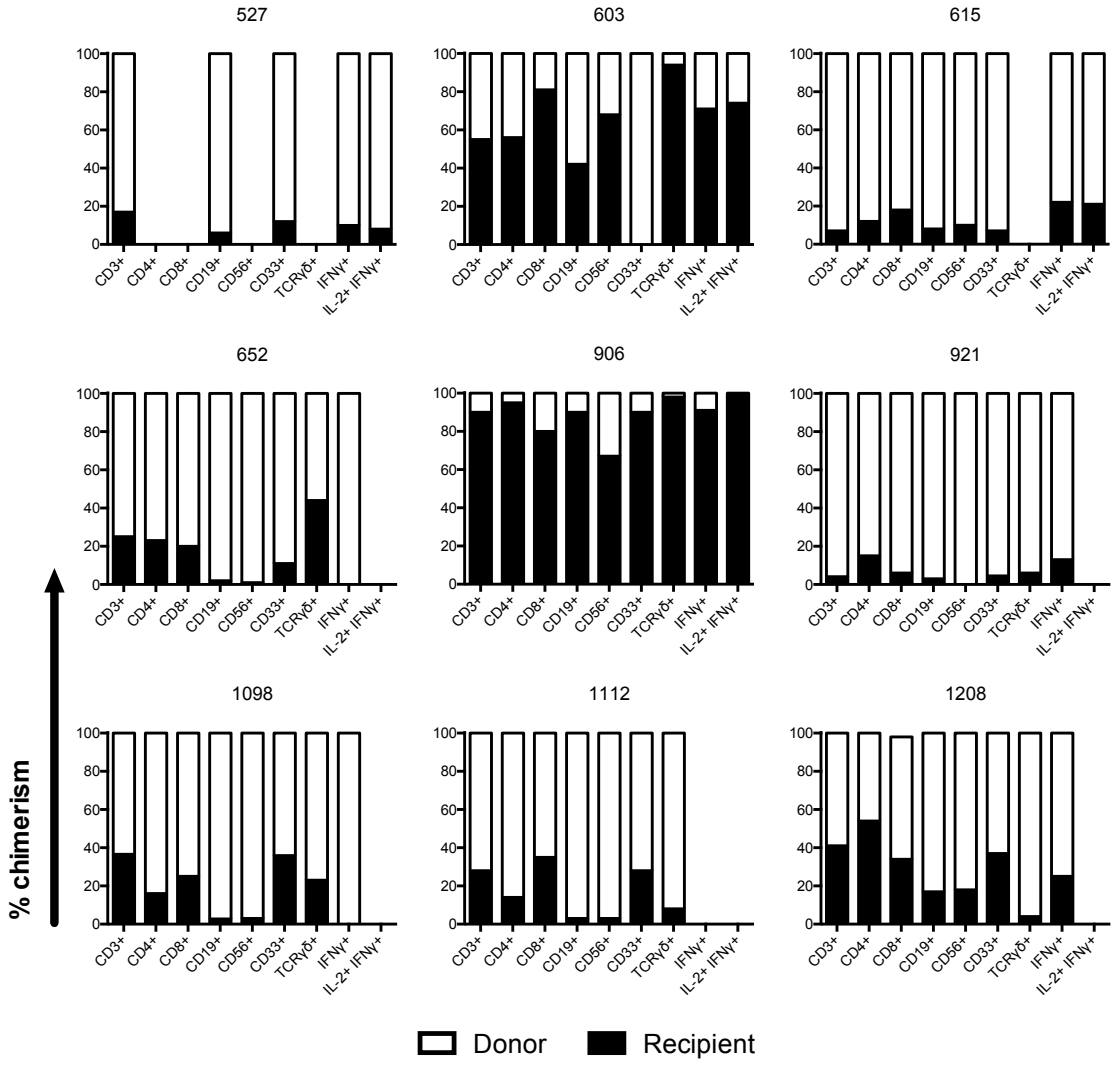

Figure 24. Chimerism results at time of inclusion for additional cellular subsets.

It would appear that not only are recipient cells present in most cellular subsets, they are also functional. One could still argue that the subsets we assessed are quite big and encompass many smaller cellular subsets. It is possible that smaller subsets are completely recipient or donor. To ascertain this, a follow-up study is needed wherein we could stimulate the cells with other substances, for instance viral antigens or E. coli. Additionally, we could try and sort for more special cell subsets, such as MAIT and NKT cells.

To conclude this section on the special phenomenon of tolerance between graft and the recipient, I feel that I need to stress again that this research was performed on small groups and on limited samples. These patients are rare and in good shape. As such they do not need regular check-ups and we had to ask them for samples specific for this study.

That being said I feel that this study has improved the knowledge on MC development. If nothing else, we proved that these patients are in good health post-HSCT and are not negatively affected by having two hematopoietic systems. Moreover, it seems that both systems are active and capable of responding to stimuli. I propose that these patients may need monitoring to ensure they do not reject the graft, especially during the first months post-HSCT. However, a stable MC in patients with non-malignant disorders may not always require intervention. As discussed, DLIs increase the risk for GVHD, which is not desirable for non-malignant disorders. In fact, some of the patients with MC in this study received DLIs shortly post-HSCT, however, they remained mixed chimeras. Hence, we conclude that MC may not be the unequivocal villain as it is sometimes seen as in the HSCT field. In some patients, it may be considered a vigilant friend.

5 CONCLUDING REMARKS & FUTURE PROSPECTS

For a successful HSCT, donor graft- and recipient-derived cells need to learn to tolerate each other. Most importantly, the donor-derived cells must learn to tolerate recipient tissue. In some cases, this learning process does not go smoothly and GVHD develops. In other exceptional cases, a long-term stable MC develops wherein not only do the donor-derived cells tolerate the recipients' cells, recipient-derived hematopoietic cells also tolerate the donor graft.

In this thesis, I tried to elucidate some of the mechanisms of these immunologic events post-HSCT and especially in the case of GVHD, to identify potential predictive and diagnostic biomarkers.

5.1 SPECIFIC CONCLUSIONS

- Clinically, predictive biomarkers for grade II-IV acute GVHD are needed. Most research groups have focused on markers in patient specimens. While difficult, I show that it is possible to identify potential predictive markers in the donor grafts.
    - Patients with grade II-III aGVHD received donor grafts with lower frequencies of MAIT cells.
    - In grade II-III aGVHD development there is a potential role for high frequencies of PD-1 and low frequencies of CD127-expressing T cells in the donor graft.
    - Increased levels of TNFα in both patient blood and the donor graft could be linked to grade II-III aGVHD development.

- Similar to acute GVHD, there is a clinical need for diagnostic biomarkers for severe grades of chronic GVHD. Identifying biomarkers for chronic GVHD development using standard laboratory techniques is difficult.
    - Patients with pronounced severity of chronic GVHD appear to have less MAIT cells in the peripheral blood.
    - New multidimensional techniques such as mass cytometry can discover novel cellular subsets (B and NKT) that may play a role in chronic GVHD.
    - Cellular subsets identified by mass cytometry can be gated for in conventional flow cytometry panels.

- Long-term stable MC in patients transplanted for non-malignancies is a rare occurrence post-HSCT. However, understanding the phenomenon may be crucial for early post-HSCT care as well as for pre-transplant treatment of live solid organ transplant recipients.
    - Donor grafts derived from sibling donors are more likely to result in stable long-term MC development than grafts from unrelated donors.
    - Long-term stable MC does not appear to be detrimental for the patients in the long run.
    - Recipient-derived cells appear to be present in most of the main immune cell subsets and are capable of responding to stimuli.

5.2 FUTURE STUDIES

Apart from the study presented in **Paper I** on acute GVHD, the other two studies presented in this thesis were performed on relatively small group sizes. Hence, conclusions must be made with caution. Additionally, all studies in this thesis were performed in a single centre setting. These findings need to be confirmed in other cohorts, preferably in other centres as well as in new cohorts within our centre.

As in most research, the studies performed in this thesis may have clarified some issues, but they do also raise a number of questions. I will discuss some of the things I would like to do in future studies.

In **Paper I**, one of the main difficulties I faced was to include patients who later developed grade III-IV aGVHD. As this study was a prospective study, I included all patients and donors I could at time of transplantation. Unfortunately, due to logistics and chance I was unable to include some patients who developed grade III-IV aGVHD. Additionally, occurrence of grade III-IV aGVHD dropped more than expected during the inclusion period. Due to these difficulties, I could not analyse patients with grade II aGVHD separately from patients with grade III-IV aGVHD.

Another issue that arose during the experimental set-up and analysis was limited access to patient samples. I was thus restricted in performing some of the experimental parts of the study.

Therefore, I would like to analyse a new cohort of patients and their donors. In this new cohort, I would then focus primarily on the identified markers of interest (PD-1, CD127 and TNFα) to validate these as potential biomarkers. Additionally, by limiting the markers of interest I could also reduce the number of flow cytometry panels before MLR and post-MLR. This will save sample and allow me to analyse cells at several time points during the MLR. This will hopefully tell us more about the activation peaks of cellular subsets, which in turn may tell us something on the *in vitro* model of aGVHD.

In a new cohort of patients, I could also potentially try a different *in vitro* method for aGVHD. By using a confocal microscope and time-lapse imaging, you can track killing and migration of single cells over time. Target and effector cells can be labelled in different colours and specific killing can be monitored. I had initially hoped to use this method in this study but it was not possible due to high spontaneous death of cells after thawing. I hope this issue can be resolved so I can try out this method in the setting of aGVHD.

Lastly, there are few things I have not yet analysed in context of the study. For instance, I also collected cells prior and after MLR for TCRγδ spectra-typing. This data is currently being analysed. Moreover, in this study I only looked at one primary outcome, e.g. primary aGVHD development. I am currently further analysing the data and looking at how donor graft phenotype may influence other HSCT outcome variables, such as; relapse, rejection, infections, engraftment, cGVHD and overall survival.

In **Paper II**, I first attempted to identify diagnostic markers for chronic GVHD through standard methods as tried by several before us. Though I could identify some cellular subsets of interest (MAIT cells) I quickly concluded I would need to utilize multi-dimensional methods to identify novel subsets linked to cGVHD severity. Due to sample availability and financial limitations, I could only analyse small group sizes (around 10 patients per grading) by mass cytometry. I then used a supplementary cohort to validate the mass cytometry in flow.

There were a few things I wanted to do in this study but could not. One of the first things was that I only had access to patient peripheral blood. However, to understand the pathophysiology of cGVHD, blood will only yield a limited amount of information. As cGVHD occurs primarily in the affected tissues, it would be interesting to analyse biopsies taken from patients suspected to suffer from cGVHD. In an ideal world, we can compare affected and unaffected tissue samples from the same patient and organ with their blood samples. I could then perhaps ascertain if there are differences in the immune phenotypes of infiltrating cells and whether an influx of MAIT cells from the blood towards affected organs could be seen. The logistics of this was unfortunately not possible for the scope of this thesis. Though not planned yet, perhaps it will be possible to analyse such biopsies in the future, though it might be difficult to obtain ethical permission especially for biopsies of unaffected tissue.

In a new study with a new cohort of patients with cGVHD, I would like to focus on some of the novel cellular subsets identified in this study and elaborate on them by incorporating other cellular markers. This might help explain some of the mechanisms of cGVHD.

Additionally, in a future study, it would be important to include patients with moderate and severe cGVHD who are untreated with immunosuppressive drugs for many months/years. I would then be able to compare patients with all cGVHD gradings to each other, which was not possible in this study. I could identify far fewer differences between moderate and severe cGVHD than between patients without cGVHD and mild cGVHD, which most likely was due to the immunosuppressive regimen.

Moreover, as these patients may present with different organs affected by cGVHD, perhaps it would be better to compare them according to affected organ. For instance, the blood immune phenotype of patients with lung-affected cGVHD could be vastly different from those that do not have their lungs affected. I am currently analysing both flow and mass cytometry data according to the organ involvement for patients with moderate and severe cGVHD.

Another way to compare the cGVHD patients could be by their ability to react to stimuli. I am currently also analysing mass cytometry data of the patients with cGVHD after PMA stimulation.

In **Paper III** and **IV**, I focused on patients with long-term stable MC. Long-term stable MC is not a common occurrence, made even more complicated by our stringent inclusion criteria of being at least 5 years post-HSCT and transplanted for non-malignancies, resulting in a small sample size.

We are currently discussing a follow-up clinical-oriented study on additional MC patients. It has been almost 5 years since I included the patients for this study and quite a few more patients with long-term stable MC have been identified. Moreover, as far as I am aware, all patients with long-term stable MC discussed in this study are still alive and doing well at time of writing this thesis. Hence, I would most likely be able to recruit a much larger group of patients in such a new study.

I could then also perform chimerism analyses on more in-depth cellular subsets. This will help identify whether these patients retain functional recipient cells in smaller immune cell subsets or whether some are predominantly donor-derived. For instance, by exposing the patient's lymphocytes to varying antigens I could ascertain whether the different hematopoietic systems react to the same extent or not.

Unfortunately, increasing the group size in a new study will not fix the potential selection bias I had in this study. Since I only included patients who survived until at least 5 years post-HSCT I may have a survival bias. A prospective study might alleviate some of this potential bias. I could then also better analyse the role of IgG3, platelets, IL-4 and ZAP-70 deficiency in MC development.

In short, we are left with perhaps more questions after the research is done than we had when we started. A lot of work is still needed and much more research needs to be performed to validate our findings. However, I feel that the research performed for this thesis is a good starting point for many more follow-up studies which I hope will be done in the near future.

6 POPULAR SCIENTIFIC SUMMARIES

6.1 SVENSKA

Vem är jag? Vad är "själv"? Var slutar jag och börjar du?

Dessa frågor har sysselsatt filosofer i århundraden. Intressant är att vi alla har ett system inom oss som syftar till att svara på dessa frågor på daglig basis: immunsystemet.

Våra kroppar attackeras dagligen av organismer som kan orsaka skada eller sjukdom. För att bekämpa detta är ett avancerat försvarssystem som heter immunsystemet aktivt dygnet runt. Immunsystemet består av ett omfattande nätverk av celler som har förmåga att kommunicera och utveckla lämpliga försvarsstrategier. För att immunförsvaret ska kunna skydda oss måste det först kunna identifiera "själv" från "icke-själv". Jämför detta med en militär, som måste kunna identifiera vän från fiende för att förhindra vådaskjutning.

Hos vissa människor fungerar inte immunsystemet lika bra som det borde. Detta kan till exempel bero på genetiska orsaker eller på grund av cancer i immunsystemets celler. Patienter som lider av ett icke-funktionellt immunförsvar kan behandlas genom att få ett nytt immunsystem. På liknande sätt som patienter med hjärtproblem kan få ett nytt hjärta från en donator, kan patienter få ett nytt immunsystem från en donator. Att donera ditt immunförsvar är något du gör medan du lever, eftersom endast en liten del av immunsystemet transplanteras. Denna del av immunsystemet kommer då att växa inuti patienten tills ett funktionellt immunsystem har återbildats. Denna typ av transplantation kallas för hematopoetisk stamcellstransplantation.

Transplantation av immunsystemets celler är i de flesta fall det enda sättet att rädda dessa patienters liv. Denna behandling har funnits länge men det finns fortfarande ett antal problem som kan uppstå för patienter efter transplantationen.

Till exempel, eftersom cellerna i det nya immunsystemet fortfarande är relativt få vid transplantation behöver de tid att växa och expandera. Under denna period av tillväxt är immunförsvaret inte fullt kapabelt att reagera på ett hot. Således kan en förkylning vara ganska problematisk för dessa patienter. Patienterna måste vara försiktiga och ta mycket förebyggande läkemedel.

Ett av immunsystemets stora problem, som kan uppstå efter en hematopoetisk stamcellstransplantation, handlar om problemet med att identifiera "själv". Per definition är patientens kropp full av celler som inte är "egna" för donatorns immunsystem. Om man inte gör någonting åt detta, förväntas det nya immunsystemet från donatorn känna igen patientcellerna som "icke-själv" och därmed reagera. Detta kallas "graft-versus-host" sjukdom. Graftet (donator immunsystemet) står motsatt host (patientens kropp) och ett krig bryter ut. Två av de fyra artiklar (**artiklar I och II**) som diskuteras i denna avhandling handlar om detta problem efter transplantation. I dessa artiklar har vi försökt hitta mätbara markörer hos patienter eller givare som kan hjälpa oss att förutsäga eller diagnostisera "graft-versus-host" sjukdom hos patienter.

I allmänhet är målet med en hematopoetisk stamcellstransplantation att ersätta patientens immunförsvar helt. I sällsynta fall kvarstår en del celler från patientens eget immunsystem och är fortfarande närvarande tillsammans med givarens immunsystem, även många år efter

transplantationen. Dessa patienter har i huvudsak två immunförsvar, sitt egna och givarens. Detta kallas blandad chimerism. De två immunförsvaren har gjort något anmärkningsvärt, de har lärt sig att inte reagera på varandra och på ett sätt identifiera varandra som ”själv”. De två sista artiklarna (**artiklar III och IV**) handlar om denna blandade chimerism. Vi försökte ta reda på hur dessa patienter mår och hur deras immunsystem fungerar.

”Graft-versus-host” sjukdom och blandad chimerism är två olika vägar som immunceller kan ta i en patient efter en hematopoetisk stamcellstransplantation. I det första fallet upplever donatorn och patienten varandra som ”icke-själv”, de är fiender och ett krig bryter ut. I det senare fallet har givare och patient funnit ett sätt att samexistera, tolerera varandra och att se varandra som ”själv” och fred råder. Vad är det i patienten som styr att det blir den första eller den andra vägen? Är det möjligt att förutspå i vilken riktning det kommer att gå, krig eller fred? Och vilken betydelse har detta för patientens välfärd? Dessa är några av de svåra frågor som uppkommer vid ”graft-versus-host” sjukdom och blandad chimerism, vilka jag har försökt att besvara i denna avhandling.

6.2 NEDERLANDS

Wie ben ik? Wat is het “zelf”? Waar eindig ik en begin jij?

Deze vragen hebben filosofen eeuwenlang geplaagd. En dat terwijl we allemaal een systeem in ons lichaam hebben dat dagelijks deze vragen moet beantwoorden: het immuunsysteem.

Onze lichamen worden continu aangevallen door organismen die schade of ziekte veroorzaken. Om dit te bestrijden, is een ingewikkeld verdedigingssysteem 24 uur per dag actief, namelijk het immuunsysteem. Het immuunsysteem bestaat uit een uitgebreid netwerk van cellen die de mogelijkheid hebben om verdedigingsstrategieën te communiceren, aan te passen en te ontwikkelen. Maar wil het immuunsysteem ons goed beschermen, dan moet het eerst het ”zelf” van het ”niet-zelf” kunnen onderscheiden. Vergelijk dit met een soldaat, die een bondgenoot moet kunnen herkennen, om eigen vuur te voorkomen.

Bij sommige mensen vertoont het immuunsysteem gebreken. Dit kan bijvoorbeeld een genetische oorzaak hebben of veroorzaakt worden door een vorm van kanker in het immuunsysteem. Patiënten die een ineffectief immuunsysteem hebben, kunnen behandeld worden met een transplantatie waarbij ze een nieuw immuunsysteem ontvangen van een donor. Het behandelingsprincipe is enigszins te vergelijken met een harttransplantatie, waarbij patiënten met hartproblemen een nieuw hart van een donor ontvangen. In tegenstelling tot een hart kun je je immuunsysteem doneren terwijl je zelf nog leeft, omdat slechts een klein deel van het immuunsysteem getransplanteerd wordt. Dit kleine deel van het immuunsysteem groeit dan in de patiënt uit tot een volledig functioneel immuunsysteem. Deze transplantatie wordt een hematopoietische stamceltransplantatie genoemd.

De transplantatieprocedure is in de meeste gevallen de enige manier om het leven van dergelijke patiënten te redden. De procedure bestaat al lang, maar helaas zijn er nog altijd problemen die kunnen optreden na de transplantatie.

Zo heeft het nieuwe immuunsysteem tijd nodig om te groeien. Gedurende die periode kan het nog niet goed reageren op een bedreiging in de vorm van een schadelijk organisme. Zo kan zoiets als een simpele verkoudheid bij deze patiënten voor grote problemen zorgen. Hierdoor moeten patiënten voorzichtig zijn en veel voorzorgsmaatregelen nemen.

Eén van de belangrijkste complicaties die zich kan voordoen na een hematopoietische stamceltransplantatie draait om het probleem van de identificatie van het “zelf”. Het lichaam van de patiënt zit vol met cellen die ”niet-zelf” zijn voor de immuuncellen van de donor. Zonder ingrijpen zal het nieuwe immuunsysteem de cellen van de patiënt bestempelen als "niet-zelf" en de cellen gaan aanvallen. Dit heet graft-versus-host reactie. De graft (het immuunsysteem van de donor) staat tegenover de host (het lichaam van de patiënt) en een oorlog breekt uit. Twee van de vier artikelen (**artikel I en II**) die in dit proefschrift worden besproken gaan over dit probleem. In deze artikelen hebben we geprobeerd markers te vinden die we kunnen meten bij patiënten of donoren, die ons kunnen helpen bij het voorspellen of diagnosticeren van patiënten die aan deze complicatie lijden.

In het algemeen is het uiteindelijke doel van een hematopoietische stamceltransplantatie dat het immuunsysteem van de patiënt volledig vervangen wordt. Er zijn echter zeldzame

gevallen, waarbij een deel van het eigen immuunsysteem van de patiënt aanwezig blijft. Deze patiënten hebben in wezen twee immuunsystemen, hun eigen en dat van de donor. Dit heet gemengd chimerisme. De twee immuunsystemen hebben iets opmerkelijks gedaan, ze hebben ogenschijnlijk geleerd elkaar te tolereren. De laatste twee artikelen (**artikel III en IV**) van dit proefschrift gaan over dit gemengd chimerisme. We hebben onderzocht hoe deze patiënten eraan toe zijn en hoe hun bijzondere mengeling van immuunsystemen functioneert.

Graft versus-host reactie en gemengd chimerisme zijn twee verschillende paden die de immuuncellen binnen een patiënt kunnen bewandelen na een hematopoietische stamceltransplantatie. In het eerste geval herkennen de cellen van de donor de cellen van de patiënt als ”niet-zelf” en een oorlog breekt uit. In het tweede geval hebben de immuuncellen van de patiënt en de donor geleerd elkaar te tolereren, waardoor er een vorm van vrede ontstaat. Wat gebeurt er binnen de patiënt dat bepaalt of het ene pad of het andere pad bewandeld wordt? Hoe kunnen we van tevoren voorspellen welk pad bewandeld gaat worden? En wat betekent dit voor het welzijn van de patiënt? Dit zijn een paar van de vragen omtrent graft-versus-host reactie en gemengd chimerisme, die dit proefschrift heeft getracht te beantwoorden.

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