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GVHD (graft-versus-host disease) prediction and diagnosis through molecular methods in HSCT (hematopoietic stem cell transplantation)

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Contents

1 Summary

Hematopoietic stem cell transplantation (HSCT) is a commonly used therapy in patients with hematopoietic disorders, when confentional chemotherapy is no possible treatment or has not been effective. The main complication after HSCT besides relapse is graft-versus-host disease (GVHD), where immunocompetent donor T cells can recognize recipient cells as foreign through differences in their human leukocyte antigens (HLA) and thus start an immune reaction against the recipient. GVHD leads to tissue damages, whereas thymus, secundary lymphoid tissues, skin, liver and intestine are primarily affected, and can also cause death. Thus, it is of interest to diagnose GVHD as soon as possible to be able to start an early treatment and to prevent a further progress of the disease. Nowadays GVHD is diagnosed only on clinical symptoms.

The aim of our study is to find molecular markers for diagnosing and predicting GVHD before symptomes occur as well as to be able to ameliorate the outcome of HSCT in further studies by downregulating GVHD development through influencing regulatory molecules.

In our study 76 patients with a median age of 45, who applied allogeneic HSCT were included. Leukemia and myeolodysplastic syndromes (MDS) were the most common diagnosed diseases in those patients. Bone marrow stem cells (BMSC) and peripheral blood stem cells (PBSC) were the main stem cell sources used for HSCT, whereas only 7 patients applied cord blood stem cells (CBSC) as graft. We analyzed the gene expression levels of IFN-γ, IL-10, IL-18, ICOS, Foxp3, FuT7 and B7-int in $CD4^+$ and $CD8^+$ T cells before HSCT and at different timepoints after HSCT as well as the donors' gene expression levels with real time quantitative-PCR (RQ-PCR) and measured also the donors' and patients' (different timepoint occassions) cytokine protein levels of IFN-γ, IL-10, IL-18, IL-12, IL-17 and IL-23 in the plasma with enzyme-linked immunosorbent assay (ELISA). The gene expression and protein plasma levels were analyzed to find a correlation between those levels and the appearance of GVHD.

In conclusion, IFN-γ could be used as a predicting molecular marker for aGVHD development, whereas IL-18, Foxp3, FuT7 and ICOS are possible diagnostic markers. Higher IFN-γ plasma levels (> 200 pg/ml) at the day of HSCT ($p = 0.009$) are correlated with a decreased aGVHD outcome, whereas IL-18 plasma levels are increased at the onset day of aGVHD ($p < 0.001$). Foxp3 ($p = 0.005 – 0.020$), FuT7 ($p = 0.001 – 0.023$) and ICOS ($p = 0.005 – 0.029$) gene expression levels are enhanced at different timepoints after HSCT in case of GVHD.

IFN-γ may be a key cytokine which production intervention could probably lead to a reduced occurrence of aGVHD.

2 Aim of the study

The overall aim of the study is to find a correlation between cytokine plasma levels as well as T cell gene expression levels and the appearance of GVHD after HSCT.

Some specific aims are:

- To predict GVHD before symptoms occur.
- To be able to start an earlier treatment of GVHD through molecular diagnostic markers, thus ameliorating the survival rate after HSCT.
- To find key cytokines and regulatory molecules in GVHD that theoretically could be influenced to downregulate the occurrence of GVHD.

3 Introduction

3.1 Preface

In our study patients with different hematopoietic disorders applied hematopoietic stem cell transplantation (HSCT) as treatment. Thus an overview of HSCT and its complications focusing on graft-versus-host disease (GVHD) and the main appearing hematopoietic diseases is given in the following section. Furthermore the measured genes and cytokines that may play a crucial role in the development of GVHD are explained.

3.2 Hematopoietic Stem Cell Transplantation (HSCT)

The first bone marrow transplantation (BMT) was accomplished in 1939. The idea was to cure aplastic anemia by injecting human bone marrow cells to the patient which should lead to reestablishing of a normal blood cell level. But it was without success (Léger and Nevill, 2004). In 1957 Thomas Donnall, who got the Nobel Prize for his work, ran the first BMT on a leukemia patient, who got irradiated before the transplantation. The BMT led to newly matured blood cells, but the patient died in the end (Barkholt and Ringdén, 2004; Thomas, et al., 1959). Georges Mathé succeeded BMT to patients, whose bone marrow was damaged due to a reactor accident and have recovered their own bone marrow after transplantation. Through further studies Thomas Donnall improved BMT and its break through came in the 1970s through the identification of the human leukocyte antigens (HLA) system, leading to transplantations of HLA receptor-identical donors (Barkholt and Ringdén, 2004; Gatti, 1968).

Nowadays HSCT is applied to many patients with hematological malignancies, non-malignant bone marrow disorders and genetic diseases associated with abnormal hematopoiesis and function (Uzunel, 2003). The term BMT is meanwhile less commonly used and often replaced through HSCT, because bone marrow is not the only stem cell source used in transplantations nowadays. The aim of the transplantation is that the abnormal hemolytic system is restored with the healthy donor system as donor cells are taking over the blood cell production.

HSCT is divided into allogeneic, autologous and syngeneic transplantations. Allogeneic implies that the stem cells are taken from a not genotypical ident donor, like a HLA-ident sibling or a HLA-like donor (Barkholt and Ringdén, 2004; Léger and Nevill, 2004). In the case of an autologous HSCT the patient gets its own stem cells, which were taken before conditioning and properly manipulated. The main risk factor of autologous HSCT is that tumor cells remain after their abatement. But finding a HLA-matched donor for allogeneic

HSCT can be complicated. HLA-identical siblings are only available in about 30% of the cases. HLA-matched unrelated donors (MUD) are disposable with a 60-90% probability depending on the patient's origin. A syngeneic HSCT is done very rarely, because a monozygotic twin is needed as donor. A huge advantage is though that no immunosuppression is needed (Barkholt and Ringdén, 2004).

3.2.1 Conditioning

Conditioning is a pre-transplantation treatment with two main aims: the elimination of the cancer cells and the suppression of the immune system, so that the graft will be engrafted and not rejected (Barkholt and Ringdén, 2004; Léger and Nevill, 2004; Uzunel, 2003). There are different types of conditioning regimes. If the recipient is in quite good health, showing no comorbidities, do not have a fast proceeding disease, and is neither too old (>60 years) nor a young child, the myeloablative conditioning is generally the method of choice (Barkholt and Ringdén, 2004; Léger and Nevill, 2004; Sala-Torra et al., 2008). In this conditioning regime chemotherapy (with high-dose cyclophosphamide application) with or without combination of total body irradiation (TBI) is applied (Barkholt and Ringdén, 2004; Fefer et al., 1974; Léger and Nevill, 2004). In case of hematological malignancies it is important that the abnormal cells are eliminated, reached through chemotherapeutic agents that inhibit cell proliferation and through radiotherapy, whereas the bone marrow that will be replaced by the donor's one, is usually additionally irradiated. Accessorily the immune system has to be restrained via conditioning, like in non-malignant hematological diseases, so that the stem cell transplant will not be rejected (Barkholt and Ringdén, 2004; Léger and Nevill, 2004; Storb and Thomas, 1983; Thomas et al., 1975). TBI can also be fractioned into different sub-irradiations, leading to a higher total dose rate in the end, which is seen to minimize the risk for severe complications in some diseases, but not in leukemia (Barkholt and Ringdén, 2004).

If the patient's conditions are not appropriate to the myeloablative conditioning, like in case of higher age or organ failure, a milder form, the so-called non-myeloablative or reduced-intensity conditioning (RIC) is adopted (McSweeney and Storb, 1999; Sala-Torra et al., 2008; Slavin et al., 1998, Welniak et al., 2007). The aim of this method is that not all tumor cells are getting destroyed through the conditioning, using *"lower doses of cytoreductive treatments and incorporating more directed immunosuppressive agents" (Welniak et al., 2007)* (Barkholt and Ringdén, 2004; Mohty et al., 2005; Sala-Torra et al., 2008). The remaining malignant cells should be eliminated by the donor's stem cells.

If the patient suffers from a severe immunodeficiency, no conditioning is needed, because the immune system is not able to reject the transplant (Barkholt and Ringdén, 2004).

3.2.2 Stem Cell Sources

There are three main stem cell sources used for HSCT: bone marrow that is either taken from the spinal cord or the pelvic bone, peripheral blood and umbilical cord blood (UCB) (Gluckman et al., 1997; Kurtzberg et al., 1996; Welniak et al., 2007). Nowadays the stem cell harvesting from peripheral blood is more common than the *"direct aspiration from the bone marrow" (Léger and Nevill, 2004)*. For peripheral blood stem cell (PBSC) harvesting the donor has to be treated with hematopoietic growth factors, granulocyte colony-stimulating factor (G-CSF) or granulocyte macrophage colony-stimulating factor (GM-CSF), respectively so that the stem cells proliferate and circulate into the periphery (Barkholt and Ringdén, 2004; Léger and Nevill, 2004; Schmitz et al., 1995). The stem cells can then be isolated from the donor's blood via their CD34 surface marker (Barkholt and Ringdén, 2004). A big advantage of the peripheral blood as stem cell source is that neutrophils and platelets recover faster after the engraftment as with bone marrow or cord blood HSCT, and more stem cells can be gained (Bensinger et al., 1993; Welniak et al., 2007). It is better if more stem cells can be transplanted, because the engraftment will be faster and the risk for rejection or infections is lowered (Deeg et al., 1985; Paulin et al., 1987). Umbilical cord blood as stem cell source has the advantage that it can act as a more tolerant immune system, because it is more immature, leading to a lower GVHD risk but otherwise to an enhanced rejection that is maybe due to the low cell amount (Gluckman et al., 1997; Kurtzberg et al., 1996; Wagner et al., 1995). In the 1990s umbilical cord blood was only used in children, based on its low stem cell amount, but nowadays it is also applied in adults by transplanting two cord bloods from unrelated donors or by in vitro expansion of cord blood cells (Léger and Nevill, 2004; Laughlin et al., 2004).
The transplanted stem cells *"home to the bone marrow" (Léger and Nevill, 2004)* in the patient, where they start to re-establish the hematopoiesis. During the time period from conditioning to engraftment, when a normal blood cell amount and immunoreconstitution is reached, the recipient's immune system is very weakened and coincides with an enhanced infection risk that is diminished by antifungal and antiviral prophylaxis (Barkholt and Ringdén, 2004; Léger and Nevill, 2004). Until the immune system is fully recovered with normal T and B cell activity it takes about one year (Lum, 1987).

3.2.3 Complications

The most common complications relating to HSCT are GVHD, relapse and graft failure or rejection, but also *"organ damage, blood vessel damage, cataracts, secondary cancers and death" (Barkholt and Ringdén, 2004)* can occur (Uzunel, 2003). Organ and cell damage is often due to conditioning, whereas secondary cancers can occur, if not all malignant cells have been deleted leading to metastasis forming and tumor cell dissemination. A high risk factor of stem cell transplanted patients is their impaired immune system that can cause severe infections, which can lead to death (Welniak et al., 2007). Most infections are effected by host's own microorganisms and the activation of herpes simplex virus (HSV) or cytomegalovirus (CMV) (Barkholt and

Ringdén, 2004).

Host-versus-graft (HVG), or rejection is a disease, where the immune reaction is caused by remaining recipient immune cells that can recognize the graft leading to graft rejection, occurring at about 10% of HSCT (Uzunel, 2003). Different host cells like *"NK cells, NKT cells, γδ T cells, and/or $CD4^+$ and $CD8^+$ T cells" (Welniak et al., 2007)* recognizing donor's HLA can mediate rejection (Haraguchi et al., 2005, Welniak et al., 2007). Graft rejection is primarily seen by transplants of MUDs and T cell-depleted grafts (Welniak et al., 2007).

Relapse, where the disease recurs after applied HSCT, appears more frequently in autologous than in allogeneic HSCT, because the positive graft-versus-leukemia (GVL) effect is missing (see page 13). But relapse can also occur after allogeneic HSCT. In case of early relapse the mortality rate is high, whereas patients with later relapse may undergo additional chemotherapy with second HSCT, although the outlook for a long-term survival is also low in this relapse group, due to the toxicity of a further treatment (Mortimer et al., 1989; Radich et al., 1993; Uzunel, 2003).

3.3 Graft-versus-host disease (GVHD)

GVHD, an analogous disease to rejection, is the *"main reason for transplant-related mortality (TRM)" (Uzunel, 2003)* and thus the major hindrance for a positive HSCT outcome (Anderson et al., 2008; Fujimori et al., 2000; Uzunel, 2003). GVHD occurs, when immunocompetent graft T cells differ from the recipient's minor histocompatibility antigens (MiHAs) or HLA and thus can recognize them as foreign and start an immune reaction against the recipient (Ferrara, 2007; Krenger et al., 1997; Reddy and Ferrara, 2003). The recipient cells are destroyed by this immunological reaction and its immune system is suppressed through conditioning and thus cannot innitate an immune response against the allogeneic donor cells (Abbas and Lichtman, 2003: p.388)

Different factors can lead to a higher risk for developing GVHD. Higher age (>50 years) of the donor or recipient, disparities in the HLA-locus or the MiHAs between donor and recipient and also specific host alleles in the HLA-locus play crucial roles in GVHD development (Bross et al., 1984; Kollman et al., 2001; Rowe et al., 2006; Welniak et al., 2007). Male recipients with female donors have a higher risk for developing chronic GVHD (cGVHD) due to disparities between MiHAs encoded on the Y and X chromosomes, whereas they have a lower risk for relapse that is probably due toa positive influence on the GVL effect (Miklos et al., 2004; Randolph et al., 2004). Such well characterized MiHAs that are encoded by genes on the Y chromosome and induce T cell response through female transplants are for example dead box RNA helicase Y (DBY), ubiquitously transcribed tetratricopeptide repeat gene, Y-linked (UTY) and drosophila fat-facets related Y gene (DFFRY) (Miklos et al., 2004; Vogt et al., 2000; Wang et al., 1995). A female donor, who was immunized through an earlier blood transfusion or pregnancy against these antigenes, increases the risk for cGVHD in a

male recipient accessorily (Bross et al., 1984; Miklos et al., 2004; Rowe et al., 2006).
Different conditionings as well as *"the tissue source, purity and number of donor immune cells that are infused" (Welniak et al., 2007)* can affect the development of GVHD too (Barkholt and Ringdén, 2004). If killer immunoglobulin-like receptors (KIR) that can recognize HLA-alleles and its ligand exhibit disparities between donor and recipient and through the KIR's influence on NK cell activity GVHD, rejection, and also the positive graft-versus-tumor (GVT) effect can be affected (Davies et al., 2002; Farag et al., 2006). In UCB more differences in MiHAs between donor and recipient occur than in HLA-identical related bone marrow grafts, but although the risk for severe GVHD is lower. This may be explained through two effects that on the one hand cord blood stem cells (CBSC) are not that mature, thus not that affective against recipient's antigens and on the other hand MiHAs act as targets for GVT activity (Barker et al., 2001; Goulmy et al., 1983; Takahashi et al., 2007).

3.3.1 The Positive GVL/GVT Effect

A positive effect of GVHD can be the occurrence of GVL/GVT. Cytotoxic donor T and NK cells can react against leukemia or malignant cells that remained after conditioning leading to their elimination (Barkholt and Ringdén, 2004; Robertson and Ritz, 1990). TNF-related apoptosis-inducing ligand (TRAIL) that can induce caspase-8-dependent apoptosis is seen to be important for the positive GVL/GVT effect (Reddy and Ferrara, 2003). If the patient survives GVHD the probability for relapse is very low due to GVL/GVT (Weiden et al., 1981). Studies are going on with the aim to prevent the GVHD development but to maintain the positive GVL effect of donor T cells to elevate the patients' survival rate (Dazzi et al., 2000; Mackinnon et al., 1995).

3.3.2 Acute and Chronic GVHD

GVHD can be distinguished in two different diseases, acute and chronic GVHD (Welniak et al., 2007).
Acute GVHD (aGVHD) normally appears within the first 100 days after transplantation. The first organs that are affected are the thymus and secondary lymphoid tissues, the skin, the liver and the intestine, whereas also the lung can be affected (Ferrara, 2007; Welniak et al., 2007). aGVHD occurs in about 40% of HLA-identical siblings and in about 80% of unrelated donor transplants (Léger and Nevill, 2004). Four grades (I-IV) with different strength can occur, whereas grade III and IV leads to a death rate of 80%. The reason for death is often a consequence of both conditioning and GVHD that cause immunosuppression and can therefore lead to infections with opportunistic pathogens (Ferrara, 2007; Przepiorka et al., 1995). A T_H1 type immune response (IL-12, IL-2, IFN-γ) has been seen to be the main trigger in the development of aGVHD, but also T_H2 donor T cells are involved in its development (Mohty et al., 2005; Reddy and Ferrara, 2003). It is still debated how a restriction towards T_H1 or T_H2 cell response contributes to the outcome of GVHD (Murphy et

al., 1998). IL-12 induces T_H1 cells leading to monocytes and macrophages activation whereby inflammatory cytokines *"such as tumor necrosis factor-α (TNF-α), IL-1 and IL-6" (Mohty et al., 2005)* are produced. This cytokine storm can lead to the development of aGVHD (Reddy and Ferrara, 2003). But early polarization of donor T cells to T_H1 cells by host cytokines can also extenuate aGVHD, as has been shown in mice so far (Brok et al., 1993; Reddy and Ferrara, 2003).

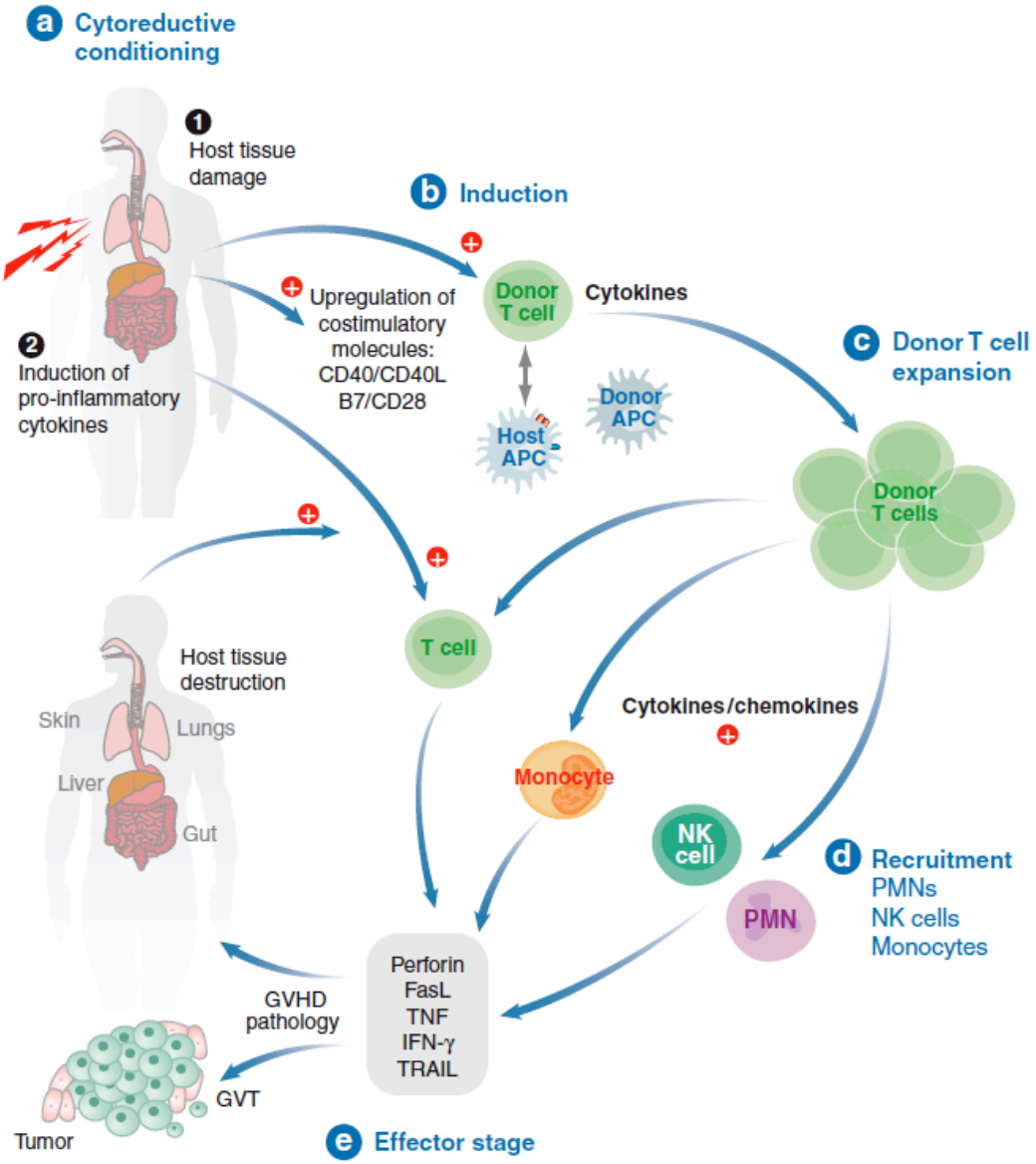
It is quite well understood how GVHD arise. Its pathophysiology is classified into three main stages (Krenger et al., 1997; Welniak et al., 2007). The pathophysiology of GVHD is shown in figure 1. The first stage primes the immune response triggered by aftereffects of the conditioning that lead to host tissue damage and distribution of pro-inflammatory cytokines, primarily TNF-α and IL-1 (Ferrara, 2007; Krenger et al., 1997). The released cytokines activate host and donor antigen-presenting cells (APCs) and *"upregulate costimulatory molecules (B7/CD28 and CD40/CD40L)" (Welniak et al., 2007).*

Through this step the second stage is induced, where the donor T cells get activated in secondary lymphoid tissues, primed through donor or host APCs (cross-presentation) by presentation of HLA and MiHA mismatched antigens (Ferrara, 2007; Krenger et al., 1997, Matte et al., 2004). If dendritic cells, the main APCs, do not get stimulated through inflammatory cytokines no T cell activation occurs leading instead to tolerance (Reddy and Ferrara, 2003). In the second stage the activated donor T cells primarily produce type I cytokines like IL-2, a T cell growth factor, and IFN-γ (Krenger et al., 1997). If IL-2 binds to CD25 (IL-2R α-chain), T cells produce more IFN-γ and IL-4, B and NK cell proliferation is enhanced as well as anti-apoptotic protein B cell lymphoma-2 (Bcl-2) is induced (Abbas and Lichtman, 2003, p.265). IFN-γ can increase GVHD development through its induction of inflammatory cytokines and nitric oxide (NO) (Teshima and Ferrara, 2002). A converse effect of IFN-γ on GVHD is debated in the discussion (see below).

In the third stage activated and differentiated donor T cells distribute phagocytes stimulating cytokines and migrate to GVHD target tissues, where T cells and stimulated mononuclear phagocytes, especially NK cells damage the tissues (Ferrara, 2007; Krenger et al., 1997; Welniak et al., 2007). Through tissue damage further inflammatory cytokines, like TNF-α, IL-1, IL-2 and IFN-γ are produced leading to enhanced homing of T cells, neutrophils and monocytes to target tissues (Reddy and Ferrara, 2003; Welniak et al., 2007). The destruction of the tissue in the effector stage, when GVHD is already developed, occurs via two main pathways. Mismatches in the HLA-A, -B or -C genes lead to lysis of the cells through the perforin/granzyme pathway by $CD8^+$ cytotoxic T cells (CTLs), whereas HLA-D mismatches cause GVHD by CTL lysing cells through the Fas/FasL pathway (Ferrara, 2007; Pinkoski et al., 2000). TNF-α is essential in the effector stage of aGVHD, by upregulating alloantigen presentation through activating dendritic cells, by inducing inflammatory cytokines and by its induction of apoptosis and necrosis (Krenger et al., 1997; Laster et al., 1988). Besides tissue damage *"transmission of infectious agents, consecutive multi-organ failure and death" (Rieger et al., 2006)* are the main consequences of GVHD (Rieger et al., 2006).

30-50% of the patients, who survive the first 3 months after HSCT, develop cGVHD (Barkholt and Ringdén, 2004). They are not obliged to have a previous aGVHD (80% have developed aGVHD), but it heightens the risk for cGVHD development as well as increased donor or recipient age and transplantation of not T cell-

depleted grafts (Atkinson et al., 1990; Barkholt and Ringdén, 2004; Storb et al. 1983). It is believed that *"new T cells produced after the donor's bone marrow has engrafted in the patient may cause cGVHD" (Barkholt and Ringdén, 2004*). The first organs that are affected by cGVHD are skin, lungs, eyes and mucosa, but also exocrine glands, gastrointestinal tract and serous membranes are targeted (Aractingi et al., 1996; Higman and Vogelsang, 2004; Welniak et al., 2007). cGVHD has the characteristics of autoimmune diseases that are linked to a damaged thymus due to conditioning and/or aGVHD and/or upper age (Teshima and Ferrara, 2002; Welniak et al., 2007). The development and progress of cGVHD are not fully understood, but it is known that cGVHD is connected with the formation of auto-antibodies, *"the expansion of auto-reactive recipient CD4⁺ T and B cells" (Pinkoski et al., 2000)*, as well as reduced donor anti-host CTL activity (Via and Shearer, 1988).



Figur 1: Pathophysiology of GVHD (Welniak et al., 2007)
APC, antigen-presenting cell; GVHD, graft-versus-host disease; GVT, graft-versus-tumor; IFN, interferon; NK cell, natural killer cell; PMN, polymorphonuclear cell; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

3.3.3 Prophylaxis and Treatment

There are different treatments for GVHD and its prophylaxis. Cyclosporine A, that acts as an immunosuppressor by inhibiting lymphocyte proliferation and the production of IL-2 and IFN-γ, with or without the combination of methotrexate that inhibits cell proliferation and growth, thus is also useful for the elimination of remaining tumor cells, are mainly used as GVHD prophylaxis after the transplantation (Léger and Nevill, 2004; Storb et al., 1989; Welniak et al., 2007). Instead of cyclosporine A and methotrexate also other immunosuppressors like antithymocyte globulins (ATG), tacrolimus or rapamycin are in common use, which all downregulate T cell activation and proliferation (Armand et al., 2008; Grosskreutz et al., 2003; Sehgal, 1998; Welniak et al., 2007).

ATG is very commonly used in combination with the conditioning regime, because it reduces the appearance of graft rejection and GVHD. ATG is a polyclonal antibody that leads to T cell-depletion in vivo, thus depletes engrafted donor T cells as well as host T cells and can also induce apoptosis of B cells, NK cells and monocytes, whereas hematopoietic stem cells (HSC) are not affected by ATG (Bacigalupo, 2005; Grüllich et al., 2009; Simpson, 2003; Waller et al., 2003). ATG is also seen to have a positive effect on engraftment and have anti-leukemic activity against certain leukemia forms (Grüllich et al., 2009).

In case of strong conditioning cyclosporine A is applied in combination with corticosteroids, like prednisolone, that also impair T cell proliferation and inhibit an inflammatory response (Forman et al., 1987). Whereas mycophenolate mofetil (MMF), another immunosuppressor, which inhibits T and B cell proliferation and also enhances engraftment, is used at transplantations with RIC (Barkholt and Ringdén, 2004; Forman et al., 1987; Mohty et al., 2007).

In high risk patients T cell-depletion of the graft can be used before the transplantation to suppress its reaction against the host cells and reduce GVHD development. 15% of patients with T cell-depleted grafts compared to 50% without T cell-depletion develop GVHD. But a T cell-depleted graft has also disadvantages, as it increases the incidence of infections, graft rejection or relapse (Bross et al., 1984; Krenger et al., 1997; Simpson, 2003). There are better results regarding rejection, if only $CD8^+$ T cells are depleted or if ATG is applied (Barkholt and Ringdén, 2004; Simpson, 2003).

As described above, the development of aGVHD is triggered by tissue damage through the distribution of pro-inflammatory cytokines. To decrease the risk for GVHD tissue damage is reduced through the application of cytoprotective agents like fibroblast growth factor-7 (FGF-7), IL-11 or keratinocyte growth factor (KGF) and also through reduced conditioning (Teshima and Ferrara, 2002; Welniak et al., 2007). It has been shown that *"TNF-α blockade during conditioning can delay the onset of aGVHD and decrease the incidence of severe diseases" (Krenger et al., 1997).*

As mentioned before, it is more common to use stem cells derived from peripheral blood for HSCT nowadays leading to a better neutrophil and platelet recreation, but the blood cells that have to be mobilized through G-CSF or GM-CSF are seen to cause a longer GVHD duration, if the disease appears, comparing to bone

marrow as stem cell source (Sala-Torra et al., 2008; Welniak et al., 2007).

Many researches are going on to protect patients from the development of GVHD, but if the disease is not preventable, good treatments have been established. aGVHD is normally treated with high-dose corticosteroids, thus inhibiting an inflammatory response and reducing the aGVHD reaction. It has a success rate of about 40% (Léger and Nevill, 2004; Ferrara, 2007). But also other immunosuppressiva are used for the treatment of aGVHD like cyclosporine A or the T cell-depleting ATG (Barkholt and Ringdén, 2004; Martin et al., 1990). CMV can be activated through the use of corticosteroids and thus ganciclovir, an antiviral medication that prevents CMV infections is additionally applied as prophylaxis in case of aGVHD and cGVHD (Léger and Nevill, 2004). Different antibiotics are also administered for preventing diverse infections. cGVHD is as well as aGVHD treated with different immunosuppressiva like steroids and cyclosporine A. To get cGVHD under control in high risk patients the use of thalidomide that decreases TNF-α secretion and is anti-inflammatory as well as lymph node irradiation is common (Barkholt and Ringdén, 2004).

3.3.4 Probably Relevant Cytokines and Molecular Markers in GVHD

3.3.4.1 Preface

It is still not fully understood which cytokines play a dominant role in the development of GVHD. Previously it has been considered that T_H1 cytokines that are involved in a cell-mediated immune response would trigger an alloreactive T cell response against the recipient and therefore leading to more GVHD development. T_H2 cytokines have been suggested to be immunosuppressive and thus believed to inhibit GVHD (Krenger and Ferrara, 1996). These findings could on the one hand be confirmed in some preclinical and clinical projects (Krenger et al., 1995; Williamson et al., 1997), but on the other hand contrary results have been seen both in murine models and patients' studies, where T_H1 cytokines could protect from GVHD development and T_H2 cytokines have augmented the GVHD outcome (Blazar et al., 1995; Sykes et al., 1990).

We were interested in studying different gene expression levels in $CD4^+$ and $CD8^+$ T cells as well as plasma levels of diverse cytokines that are likely to be involved in the development of GVHD in a huge number of stem cell transplanted patients looking for a correlation between T_H1 and T_H2 cytokine levels and the appearance of GVHD. It is not easy to diagnose and distinguish between GVHD-associated and infectious inflammations, thus a molecular marker would be helpful for diagnosing GVHD and starting an early treatment. Furthermore an intervention in the cytokine system could help to diminish the risk for the development of GVHD.

The gene expressions and plasma levels of the characteristic pro-inflammatory T_H1 cytokines IFN-γ and IL-18 were measured with real time quantitative-polymerase chain reaction (RQ-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Additionally the plasma level of the pro-inflammatory cytokine

IL-12 was analyzed with ELISA. The typical immunosuppressive cytokine IL-10 was analyzed at gene expression and protein level. Furthermore ICOS, Foxp3, FuT7 and B7-int gene expressions as well as the IL-17 and IL-23 protein expressions were studied.
The different cytokines and molecular markers are described in detail below.

3.3.4.2 Interferon-γ (IFN-γ)

IFN-γ is also known as type II or immune interferon due to its important role in preventing and controlling infectious diseases. It acts as a pro-inflammatory cytokine. IFN-γ is mainly secreted by T_H1 $CD4^+$ cells, $CD8^+$ T cells, γδ T cells, NK cells, and NKT cells after an inflammatory activation leading to cell-mediated immune response whereas mainly macrophages but also NK cells, neutrophils and CTLs get activated and proliferate (Abbas and Lichtman, 2003, p.268 et seq.; Chen and Liu, 2009; Ikeda et al., 2002). IFN-γ inhibits IL-4 synthesis and T_H2 cell proliferation by what it blocks a humoral immune response. During adaptive immune response $CD4^+$ T cells and $CD8^+$ T cells are the primarily IFN-γ producing cells. T cells get stimulated through antigens or IL-12 and IL-18 (pathway via signal transducers and activators of transcription 4 (STAT4) and NF-κB) to produce IFN-γ (Ikeda et al., 2002). NK cells are activated by tissue macrophages recognizing bacterial products and producing TNF-α and IL-12 at low levels to produce IFN-γ. IFN-γ itself stimulates macrophages (as the initial macrophage-activating factor) leading to more TNF-α and IL-12 production ending up in a positive feadback loop with more IFN-γ production and additionally, IFN-γ enhances its own mRNA expression (auto-amplification loop) (Ikeda et al., 2002; Young and Hardy, 1995).
T_H1 cells increase the IL-12 production by stimulated APCs and keep the IL-12 receptor β-chain expression on $CD4^+$ T cells up through their IFN-γ distribution (Ikeda et al., 2002). IFN-γ increases the antigen presentation through its upregulation of class I and II MHC gene expressions (Chen and Liu, 2009; Chesler and Reiss, 2002). Given that IFN-γ activates antiviral mechanisms by fending microbial infections it controls the immune system at many different sites. IFN-γ influences the cell cycle through growth and apoptosis, plays and important role in *"leukocyte trafficking and leukocyte endothelial interactions and in immune regulatory functions" (Chen and Liu, 2009)* such as supporting T_H1 cells and repressing T_H2 cells development. It modulates the antigen processing and inhibition capacity of T_H17 cells and has an inhibitory effect on the generation of memory T cells (Chen and Liu, 2009; Ikeda et al., 2002; Young and Hardy, 1995). It have been shown in mice models that IFN-γ plays a part in T cell homeostasis by erasing activated $CD4^+$ and $CD8^+$ T cells through apoptosis. (Badovinac et al., 2000; Refaeli et al., 2002).
IFN-γ suppresses immunoglobulin G1 (IgG1) and IgE production (induced by IL-4) and on the contrary enhances Ig2A synthesis (induced by lipopolysaccharide (LPS) or IL-2), although it does not cause class-switching. IFN-γ has both repressive and stimulatory effects on B cells by inhibiting LPS-induced proliferation of naive B cells, but acting anti-apoptotic on mature B cells and promoting their differentiation.

IFN-γ is suppressive for hematopoietic progenitor cells, but it enhances their development in combination with IL-3 and stem cell factor (Young and Hardy, 1995). It depends very on in which cytokine combinations IFN-γ occurs. For example IFN-γ acts as a *"survival factor for committed human erythroid and myeloid progenitor cells in the absence of other cytokines" (Young and Hardy, 1995)*. IFN-γ has been seen to inhibit graft rejection (Chen and Liu, 2009), but also the opposite effect has been observed, where *"donor T cells responding to host alloantigens release IFN-γ and IL-2"* (*Moore et al., 2001*), both belonging to the T_H1 cytokines. Through IFN-γ release pro-inflammatory macrophages and NK cells get activated. Those can then be triggered by gut bacteria or by latent infections. As a result inflammatory cytokines and active nitrogen intermediates are ejected, which lead to tissue damage (Moore et al., 2001). It has been observed that autoimmune responses can be inhibited by IFN-γ through the induced development of regulatory T cells (T_{reg}s) (turning $CD4^+CD25^-$ into $CD4^+CD25^+$ T_{reg}s) leading to an upregulated T effector cell apoptosis (Chen and Liu, 2009). As mentioned before cGVHD has characteristics of autoimmune diseases.

IFN-γ that is composed of 4 exons and 3 introns is coded on chromosome 12. The functional protein appears as a non-covalent homodimer (34KDa), whereas two IFN-γ polypeptids are antiparallel associated (Chen and Liu, 2009; Ikeda et al., 2002; Young and Hardy, 1995). The IFN-γ receptor consists of a α-chain that is coded on chromosme 6 and a β-chain, coded on chromosome 21. The α-chain is constantly expressed, the β-chain at very low levels on those cells which can react to IFN-γ (Chen and Liu, 2009; Ikeda et al., 2002). As shown in murine studies, T_H1 cells lack the β-chain leading to no response in contrast T_H2 cells express the β-chain and have a total response to IFN-γ that blocks their proliferation (Bach et al., 1995; Pernis et al., 1995).

IFN-γ binds to two α- and two β-chains. The α-chain is responsible for ligand binding and trafficking through the cell, whereas the β-chain is needed for signaling. Signaling occurs via the janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, inducing caspase-1 and Fas/FasL, which are IFN-γ/STAT1 dependent genes. STAT1 leads also to a basal caspase-1 expression in certain cells without IFN-γ (Ikeda et al., 2002). Caspase-1 turns IL-1β into an active form leading to an inflammatory cytokine (Abbas and Lichtman, 2003, p.254).

3.3.4.3 Interleukin-12 (IL-12)

IL-12 is an interleukin that is important for the interaction between innate and adaptive immunity and acts as an early pro-inflammatory cytokine. IL-12 controls inflammatory and infectious innate immune response as well as the persistence and initiation of the adaptive immune response. IL-12 is mainly distributed by phagocytic cells (monocytes, macrophages, neutrophils), B cells and dendritic cells, but also by mast cells, keratinocytes and skin Langerhans cells (Colombo and Trinchieri, 2002; Trinchieri, 1995; Watford et al., 2003). In combination with infectious agents IL-12 stimulates the IFN-γ production of NK cells and T cells, dendritic cells and macrophages leading to phagocytic cells and CTLs activation, and inflammation primarily

in the innate immune response (Colombo and Trinchieri, 2002; Trinchieri, 1995).

Microbial products can induce IL-12 production in two different ways, through toll-like receptor (TLR) signaling and through CD40-CD40L binding (T cell-dependant manner). IL-12, which is an antogonist to IL-4, mainly acts on T and NK cells via enhancing their proliferation, IFN-γ production and cytotoxic activity (CTLs and NK cells), and polarizing T cells into T_H1 cells leading to cell-mediated immunity and distribution of IFN-γ and IL-2 (Trinchieri, 1995; Watford et al., 2003). IL-12 has a further positive effect in combination with CD28 ligand expression on APC, on T cell receptor (TCR)/CD3 stimulation during antigen presentation leading to T cell proliferation and IFN-γ production. IL-12 additionally induces the production of TNF-α, macrophage colony-stimulating factor (M-CSF), IL-3, IL-8 and IL-2 by T and NK cells (Trinchieri, 1995).

In combination with IL-18 IL-12 upregulates the expression of IL18R on T_H1 cells and acts on macrophages, dendritic cells and B cells via enhancing the IFN-γ production in those cells and also in APCs (Lotze et al., 2002; Kashiwamura et al., 2002; Watford et al., 2003). IL-12 indirectly induces its own production, because IFN-γ positively regulates the IL-12 production, against it IL-10, IL-11, IL-13 as well as IFN-α and -β inhibit the IL-12 production (Watford et al., 2003). *"IL-12 increases IL-2-induced proliferation of activated T cells but mostly inhibits IL-2-induced proliferation of NK cells" (Trinchieri, 1995)*. IL-2 provides the proliferating effect that IL-12 has on activated T cells (Trinchieri, 1995).

IL-12 is a heterodimeric cytokine containing a disulfid-linked IL-12p35 subunit (α-chain) and a IL-12p40 subunit (β-chain). The subunits' protein expression is regulated independently, because the IL-12p35 subunit is coded on chromosome 3, whereas IL-12p40 is coded on chromosome 5. The IL-12p40 subunit can also appear as a free monomer or a homodimer ($p40_2$), but their functions are unknown (Colombo and Trinchieri, 2002; Watford et al., 2003). *"The p40 gene is regulated at the level of transcription and is highly inducible by microbial products"* (*Watford et al., 2003*). The p40 promoters bind many transcription factors, such as NF-κB, interferon regulatory factor-1 (IRF-1) and c-Rel. p35 is in contrast to p40 regulated transcriptionally as well as translationally. The p35 gene is constantly synthesized at low levels in unstimulated cells and in most cell types and its translation is stimulated through LPS. If a co-expression of both subunits occurs in the same cell, the biologically active p70 heterodimer is built (Trinchieri, 1995; Watford et al., 2003).

The IL-12R is primarily expressed on activated T cells and NK cells, but also on dendritic cells. Resting T cells do not express IL-12R and only T_H1 cells express both subunits. The receptor, which has three binding sides, is composed of the IL-12Rβ1 subunit binding to IL-12p40 and the IL-12Rβ2 subunit binding to IL-12p35 (Colombo and Trinchieri, 2002; Trinchieri, 1995; Watford et al., 2003). Both subunits are necessary for a high-affinity binding and the β2 subunit is essential for the signal transduction. The receptor subunits occur as dimers or oligomers on the cell surface. The signal transduction pathway takes place through a JAK2/STAT4 signaling (only IFN-γ and IL-12 activate STAT4) (Colombo and Trinchieri, 2002). The transcription factor for the IL-12Rβ2 subunit, T-box expressed in T cells (T-bet) is induced by IFN-γ (Watford et al., 2003).

3.3.4.4 Interleukin-18 (IL-18)

IL-18 is like IL-12 a pro-inflammatory cytokine, has also an IFN-γ-inducing factor and plays an important role in the *"transition from innate to adaptive immunity" (Lotze et al., 2002)* as in the defense against tumors and infections (Lotze et al., 2002; Kashiwamura et al., 2002). IL-18 is expressed in many cell types as in macrophages, dendritic cells, keratinocytes (in the differentiating skin cells), intestinal epithelial cells and in different non-immune cells in tissues with rapid cell proliferation, where its expression is not clearly understood yet; maybe IL-18 is involved in tissue regeneration and repair there (Kashiwamura et al., 2002). IL-18 induces both T_H1 and T_H2 cytokines and is therefore part of the destructive and compensatory pathways depending on its interaction with other cytokines (Kashiwamura et al., 2002; Nakanishi et al., 2001). In the presence of antigen and IL-12 it provides IFN-γ, TNF-α and IL-1 production (T_H1 cytokines) leading to distribution of *"NO and reacitve oxygen intermediates" (Kashiwamura et al., 2002)* by macrophages and neutrophils disturbing microbial pathogens and also tissues, whereas IL-18 provides IL-4, IL-5, IL-10 and IL-13 (T_H2 cytokines) and prostanoids, like prostaglandin E2 (PGE_2) secretion, in absence of IL-12, but supported through IL-2 and/or IL-3 (Kashiwamura et al., 2002; Min et al., 2004; Nakanishi et al., 2001). Prostanoids affect the production of cytokines, such as IL-12. Through T_H2 cytokine distribution T and NK cells secret IL-13 leading to suppression of inflammatory cytokines, like IFN-γ and IL-12. To induce a T_H2 cell response IL-18 probably has to induce the cyclooxygenase 2 (Kashiwamura et al., 2002). T_H2 cytokines are anti-inflammatory and therefore IL-18 is maybe involved in tissue repair and regeneration. IL-18 increases NK cell and $CD8^+$ T cell perforin-dependent cytotoxicity independently of IL-12. Additionally IL-18 can induce GM-CSF, IL-1β and IL-8, whereas IL-8 leads to neutrophil infiltration (Kashiwamura et al., 2002).

IL-18 belongs to the IL-1 cytokine family and is similar to their *"molecular structure, processing and signaling" (Reddy et al., 2003)*. However, IL-18 is located on another chromosome, on chromosome 11 and not on chromosome 2, like the other IL-1 homologous cytokines. Macrophages and dendritic cells store and produce IL-18 as a precursor form (pro-IL-18 with 24kD), which has to be processed by caspase-1 (IL-1β-converting enzyme) to allow its secretion and to form a fully function cytokine (18kD) (Lotze et al., 2002; Kashiwamura et al., 2002). It is unknown how caspase-1 gets triggered, but it is known, that NO directly inactivates it. Caspase-1 and IL-18 do both have promoter regions with *"the consensus sequence for IRF-1 and IFN consensus sequence binding protein" (Kashiwamura et al., 2002)*. Therefore, interferons probably control the induction and processing of IL-18 as well. Without caspase-1 the Fas/FasL system is involved in the processing of IL-18 (Kashiwamura et al., 2002).

The IL-18 gene is assembled of seven exons, whereas exons 1 and 2 are non-coding. There are 2 promoters lying upstream exons 1 and 2 that both have a NF-κB recognition sequence. The IL-18 gene lacks a RNA-destabilizing element that may be the reason for a long-lasting IL-18 production in inflammatory reactions. Different transcription factors are activated through IL-18. NF-κB can be activated through myeloid differentiation primary response gene 88 (MyD88) and TNF receptor-associated factor 6 (TRAF6) leading

among others to IFN-γ production. Its mitogen-activated protein kinase (MAPKinase) activation is involved in the activation of NK cells and also the transcription factors activator protein-1 (AP-1) and nuclear factor of activated T cells (NFAT) can be activated via IL-18 (Kashiwamura et al., 2002). The heterodimeric IL-18R, located on chromosome 2, is composed of a ligand-binding α-chain (IL-18Rα/IL-1 Rrp) and an associating β-chain (IL-18Rβ/AcPL) (Reddy et al., 2003; Sims, 2002). T_H1, but neither T_H2 nor naive T cells express IL-18R, which is induced by IL-12 leading to fast IL-18 response and IFN-γ production. NK cells constitutively express IL-18R leading to a fast enhancement of their cytolytic activity through binding of IL-18 (Kashiwamura et al., 2002; Watford et al., 2003).

3.3.4.5 Interleukin-10 (IL-10)

IL-10 is an immunosuppressive and anti-inflammatory T_H2 cytokine, which inhibits the activation of myeloid cells like monocytes, dendritic cells and macrophages leading to a decreased *"production of pro-inflammatory mediators" (Beebe et al., 2002)* and a reduced T cell stimulation. IL-10 downregulates the function of APCs that leads to a repression of $CD4^+$ T cells' cytokine production and proliferation. Thereby IL-10 has positive effects on $CD8^+$ T cells through its induction of their recruitment, proliferation and cytotoxic activity. *"Activation of T cells in the presence of IL-10 can induce non-responsiveness/ anergy, which cannot be reversed by IL-2" (Moore et al., 2001)*, that can result in T_{reg} production with a high IL-10 secretion and prevention of antigen specific responses (Beebe et al., 2002; Lynch et al., 2003; Moore et al., 2001).

IL-10 is produced by many different cell types, like *"T_H1 and T_H2 cells, $CD8^+$ T cells, mast cells, eosinophils, B cells, macrophages, monocytes, keratinocytes and various tumor cells" (Lynch et al., 2003)*. LPS-induced macrophages secret T_H2 cytokines like IL-10 as well as IL-5 and IL-13 (Lynch et al., 2003). IL-10 influences T_H1 cells, NK cells, granulocytes and endothelial cells negatively by inhibiting their pro-inflammatory functions through affecting their accessory cells' functions (Beebe et al., 2002; Moore et al., 2001). The inhibition of macrophages' and monocytes' functions implies an inhibition of *"monokine synthesis, NO production as well as of the MHCII complex and co-stimulatory molecules like IL-12 and CD80/CD86" (Moore et al., 2001)*. IL-10 increases B cell activity and proliferation through anti-IgM or CD40 cross-linking and activation, and B cell survival through an enhanced expression of the anti-apoptotic protein Bcl-2. Additionally, it enhances the Ig-production of naive and differentiated B cells and can lead to B cell differentiation into plasma cells through distinct circumstances and long influence. IL-10 plays an important role in isotype switching. It causes switching to IgM, IgG1, IgG2, IgG3 and IgA and in combination with IL-4 to IgG4 and IgE (Beebe et al., 2002; Moore et al., 2001).

IL-10, coded on chromosome 1, is a non-covalent associated homodimeric cytokine with two interpenetrating polypeptid chains. Its expression is regulated differently in different cell types, whereas the transcription factors Sp1 and Sp3 that are constantly expressed in many different cell types and are controlled

via posttranscriptional degradation have a main regulatory function. Different IL-10 gene polymorphisms are associated with diverse IL-10 productions of T cells and monocytes (Beebe et al., 2002; Moore et al., 2001).
The IL-10R belongs to the interferon receptor (IFNR) family, is heterodimeric and at least composed of two subunits (Beebe et al., 2002; Moore et al., 2001). The IL-10Rα/IL-10R1 subunit that is expressed on most hematopoietic cells but can also be expressed on non-hematopoietic cells is the ligand binding part that binds IL-10 with high affinity. T cell activation leads to low IL-10R1 expression, in contrast to it leads monocyte activation to high IL-10R1 expression, regarding that IL-10 inhibits both cell types. The IL-10R1 expression is induced in fibroblasts through LPS and in epidermal cells or keratinocytes through glucocorticoides. The IL-10Rβ/IL-10R2 subunit is not responsible for IL-10 binding, its function is binding of tyrosine kinase 2 (Tyk2), a JAKKinase, in the signaling complex. IL-10R2 is constitutively expressed in most tissue cells and no distinct activation in e.g. immune cells is known. IL-10R1 regulates its expression. IL-10 signal transduction occurs via phosphorylation of the tyrosine kinases JAK1 that is constitutively associated with IL-10R1 and Tyk1 that is associated with IL-10R2, respectively resulting in activation of the transcription factors STAT3, STAT1 and STAT5 (except in macrophages). IL-10 is inhibited through IFN-γ-induced STAT1 activation and phosphorylation. There are also other signaling pathways known, like the activation of NF-κB in $CD3^+$ T cells leading to inhibition of macrophage activation and monocyte production (shown in vitro). IL-10 activates NF-κB and AP-1 in $CD8^+$ T cells resulting in increased growth, differenciation and cytotoxic activity of $CD8^+$ T cells and NK cells. IL-10 can also induce Bcl-2 expression in $CD34^+$ progenitor and germinal center B cells causing survival of those cells (Moore et al., 2001).

3.3.4.6 Interleukin-17 (IL-17)

IL-17 belongs to the pro-inflammatory cytokines and induces the expression of those type of cytokines in many different cell types and tissues (Bettelli et al., 2008; Kastelein et al., 2007; Moseley et al., 2003; Weaver et al., 2007). It induces secretion of the pro-inflammatory cytokines IL-6, TNF-α, IL-1β, IL-8 and IFN-γ (Bettelli et al., 2008; Chen et al., 2007; O'Garra et al., 2008; Weaver et al., 2007). IL-17 is also known as cytotoxic T lymphocyte antigen 8 (CTLA8) or IL-17A, because it is a member of the IL-17 cytokine family. IL-17 is secreted by activated effector T cells belonging to the T_H17 cells, $CD8^+$ T cells, NK cells, γδ T cells, neutrophils, eosinophils and monocytes. T_H17 cells that are part of the adaptive immune system try to destroy extracellular bacteria and microbes that cannot be eliminated by T_H1 or T_H2 cells, but T_H17 cells can also cause tissue inflammation and autoimmunity through its IL-17 distribution (Bettelli et al., 2008; Moseley et al., 2003; Weaver et al., 2007). The T_H17 cell linage is also seen to be associated with allograft rejection as well as GVHD development (Dander et al., 2009; Kappel et al. , 2009; Weaver et al., 2007) and *"may play an important role in the homeostasis of tissues and the progression of diseases" (Moseley et al., 2003).*
IL-17 causes acute inflammation through activation and infiltration of neutrophils to sites of infections

triggered via its activation of monocytes, epithelial and endothelial cells (Bettelli et al., 2008; Kastelein et al., 2007; O'Garra et al., 2008; Weaver et al., 2007). For the development of T_H17 cells from naive $CD4^+$ T cells or dendritic cells the cytokines IL-1, IL-6 and transforming growth factor-β (TGF-β) are essential as well as the activation of the transcription factor retinoic acid-related orphan receptor γT (RORγT). IL-21 and IL-23 are involved too, whereas IL-21 is even produced by T_H17 cells. IL-1, IL-6 and TGF-β cause T_H17 cell differentiation, IL-21 their amplification and IL-23 their maintenance. IL-17 is not involved in the differentiation and proliferation of T_H17 cells (Bettelli et al., 2008; O'Garra et al., 2008; Weaver et al., 2007). *"TGF-β induces RORγT expression but, paradoxically, inhibits its transcriptional activity, thus preventing expression of IL-17" (O'Garra et al., 2008).* Through the influence of inflammatory cytokines like IL-1, IL-6, IL-21 and IL-23 RORγT gets transcriptional active leading to IL-17 expression (O'Garra et al., 2008). Not only naive $CD4^+$ T cells or dendritic cells but also Foxp3-expressing T_{reg}s can differentiate into T_H17 cells under the influence of IL-6, TGF-β and probably IL-1. TGF-β and IL-1 are both part of the innate immune system regulating the IL-17 expression as mentioned before and therefore is IL-17 seen to be an important component of the collaboration between the innate and the adaptive immune system (Weaver et al., 2007).
"Naive T cells activated in the presence of $CD4^+CD25^+$ T_{reg}s exhibite suppressed levels of IFN-γ and IL-2 but expresses high levels of IL-17" (Weaver et al., 2007). IFN-α, -β and -γ as well as IL-4 repress the development of the T_H17 cell linage in vitro (Weaver et al., 2007).
IL-17, a disulfid linked homodimer coded on chromosome 1, binds to the IL-17Rs, that are *"singel-pass transmembran proteins" (Moseley et al., 2003)* leading to an activation of NF-κB or MAPkinase that causes induction of pro-inflammatory cytokines (Moseley et al., 2003). The receptor IL-17RA is expressed at high levels on haematopoietic cells, and at lower levels on osteoblasts, fibroblats, endothelial and epothelial cells (Bettelli et al., 2008).

3.3.4.7 Interleukin-23 (IL-23)

IL-23 is mainly produced by activated dendritic cells and phagocytic cells, but also by T cells and endothelial cells. IL-23 plays an important role in the primary response to local inflammation by causing IFN-γ production of T cells and dendritic cells and triggers a T cell-dependent immunity by mainly enhancing the proliferation of memory T cells and stimulating their IL-17 production and causes neutorphils and macropahges infiltration to sides of infections (Kastelein et al., 2007; Watford et al., 2003; Weaver et al., 2007). Within a few hours after a microbial infection IL-23 is secreted causing a *"rapid IL-17 response from tissue-resistant αβ, γδ T cells and NKT cells" (Kastelein et al., 2007)* as well as TNF-α and IL-1β distribution from myeloid cells, only later $CD4^+$ and $CD8^+$ T cells are involved in the immune effector functions. In contrast it does neither stimulate naive $CD4^+$ T cells, which are missing a receptor for IL-23, nor T_H1 or T_H2 cells. IL-23 is therefore not required for the differantiation of $CD4^+$ T cells into T_H17 cells and their initial IL-17 secretion,

but is important for the maintenance and full effector function of this cell type, like IL-1 (Bettelli et al., 2008; Kastelein et al., 2007; Weaver et al., 2007). It also induces cells of the innate immune system to secret IL-1 and IL-6 causing inflammation and antibody producing B cells to proliferate (Bettelli et al., 2008). The T_H1/ T_H2 pathways shut down the IL-23/IL-17 pathway which is important to avoid autoimmune diseases (Kastelein et al., 2007).

IL-23 is a disulfid linked heterodimer and composed of the subunits p40 (like in IL-12) and p19 and its signaling is initiated through binding to its receptor that consists of two subunits, the IL-12Rβ1 chain binding to p40 and a particular IL-23R subunit binding to the p19 part (Bettelli et al., 2008; Kastelein et al., 2007; Watford et al., 2003). *"Many pathogens and TLR agonists enhance the expression of the p40, p35 and p19 subunits, resulting in the release of bioactive IL-12 and IL-23" (Kastelein et al., 2007)*. Dendritc cells are seen to cannot co-express p35 and p19 at the same cell assuming that there are subpopulations specific for p35 and others for p19. Signaling is mediated by JAK2 association to the receptor and the following STAT1, 3, 4 or 5 phosphorylation and activation leads to NF-κB signaling, whereas STAT4 is much weaker activated as through IL-12. The transcription factor RORγT is essential for IL-23R expression as well as for the expression of IL-1R1 (Kastelein et al., 2007; Watford et al., 2003). The expression of both IL-23R chains is mainly found on activated and memory T cells, T_H17 cells, NK cells and NKT cells, but at lower levels also on monocytes, macrophages and dendritic cells (Bettelli et al., 2008; Kastelein et al., 2007). The IL-23R expression in activated and memory T cells is intensely induced by IL-6 amd IL-21, whereas IL-23 by itself can increase its receptor expression *"through an autocrine or paracrine feedback loop" (Bettelli et al., 2008)*.

3.3.4.8 Inducible Costimulator (ICOS)

ICOS, which stands for inducible costimulator, because it is *"incduced on T cells after stimulation" (Abbas and Lichtman, 2003, p.172)*, plays an important role in many immune responses, where its and its ligand ICOSL expression is upregulated. Signaling through the ICOS/ICOSL pathway leads to an enhanced lymphocyte level in the lymph nodes with increased stimulated T and B cell pools (Tesciuba et al., 2008; Watanabe et al., 2008). Its pathway is also crucial for *" T_H2 cell differentiation, cell effector functions and antibody response, especially for germinal center formation and class switch recombination" (Watanabe et al., 2008)*, as has been shown in murine studies. Because ICOS and ICOSL have an enhanced expression in immune reaction in mouse models, it is assumed that their pathway is important *"to prevent dysregulation of immune responses" (Watanabe et al., 2008)*. But their downregulation is also essential for the immune system homeostasis, because a long lasting signaling leads to a defect homeostasis (Watanabe et al., 2008).

ICOS belongs to the CD28 family, which are Ig-like molecules such as CTLA-4 and programmed death-1 (PD-1). ICOS and CD28 are both inducible factors for T to B cell interaction and therefore crucial for an ideal antibody response. ICOS is only expressed on $CD4^+$ and $CD8^+$ T cells after their activation and TCR

stimulation reaching its maximum induction through an accessory CD28 signal. Its ligand expression is enhanced on APCs through different mitogenic stimuli. ICOS is primarily expressed on activated T_H2 cells that seems to be regulated by the transcription factor GATA3 rather than on T_H1 cells. However, ICOS is important for the expansion of both T_H1 and T_H2 responses. It is additionally constitutively expressed on memory $CD4^+$ T cells and on some subsets of $CD25^+CD4^+$ T_{reg}s. ICOS gene transcription may is regulated by the transcription factors NFAT and AP-1 (Tesciuba et al., 2008; Warnatz et al., 2006; Watanabe et al., 2008).
Stimulation of CD40 leads to an induction of ICOSL, in contrast are LPS and IL4 downregulating factors for ICOSL (Tesciuba et al., 2008; Watanabe et al., 2008). IL-10 and IL-17 are released after ICOS ligation and their stimulation cannot be fully replaced through CD28, if ICOS is absent. ICOS deficiency leads to the development of an antibody deficiency syndrome looking like common variable immunodeficiency (CVID) with an enormous decrease of the B cell pool, particularly of switched memory and naive B cells. In case of its deficiency germinal center formation is negatively affected too that is due to lowered IL-10 production of T cells, whereas T cells are not further affected besides a decreased IL-17 production (Tesciuba et al., 2008; Warnatz et al., 2006). Due to decreased B cell level the total number of IgG and IgA is drastically low, the IgM level is also decreased and IgE is not even detectable (Warnatz et al., 2006). This indicates that ICOS is essential for *"the maintenance of a mature B cell compartment" (Tesciuba et al., 2008)*. It is interesting that a prolonged ICOS expression have been seen to be connected with the occurrence of chronic, but not acute GVHD, in the mouse model (Watanabe et al., 2008).

3.3.4.9 β7-Integrin (β7-int)

There are two types of β7-integrins differing in their α-chain, the α4β7 subtype, also known as lymphocytea Peyer's patch adhesion molecule-1 (LPAM-1) is involved in the lymphocyte homing to mucosal sites, whereas the αEβ7 integrin is important for the lymphocyote retention in the epithelium. It has been shown in mouse models that the α- and the smaller β-chain are building a non-covalently associated heterodimer (Shaw and Brenner, 1995).
Naive lymphocytes have a homogenous moderate expression of the α4β7 subtype, in contrast 2 effector and memory celltypes concerning their α4β7 levels exist, those with low and those with high α4β7 integrin levels (Miles et al., 2008; Wright et al., 2002). The T and B cells' α4β7 integrin expression is induced through their *"activation by dendritic cells in gut-associated lymphoid tissues (GALT)" (Miles et al., 2008)*. Naive lymphocytes and lymphocytes with high α4β7 integrin expression home to secondary lymphoid tissues through their integrin binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on the cell surface of high endothelial venules (HEVs) on Peyer's patches, lymph nodes and on the intestinal lamina propria. The two Ig-like domains of MAdCAM-1 are responsible for its interaction with the α4β7 integrin leading to rolling of the integrin at high MAdCAM-1 and to a stop and go movement at lower expressions, whereby the lymphocytes can enter the mucosal sites (Miles et al., 2008; Pachynski et al., 1998; Shaw and Brenner, 1995;

Wright et al., 2002). α4β7 integrin can also bind to vascular cell adhesion molecule-1 (VCAM-1) and to connecting segment-1 region of fibronectin (CS-1/FN) instead for its main binding to MAdCAM-1. The α4β7 subtype that is expressed on most T cells in mucosal epithelial cells *"has been shown to mediate lymphocyte binding to epithelial cell E-cadherin" (Shaw and Brenner, 1995)*. The ligand E-cadherin is primarily found on epithelial cells but also on some leukocytes in the epidermis like Langerhans cells (Shaw and Brenner, 1995; Wright et al., 2002).

3.3.4.10 Fucosyltransferase VII (FuT7)

The α-1,3-fucosyltransferase VII enzyme (FuT7) plays an important role in the onset of an inflammatory response leading to leukocytes' homing to sites of infection. Its key role is assisting the synthesis of the sialyl Lewis x (sLe^x) epitope, which is an essential component of functional active ligands for E-, P- and L-selectin on leukocytes, by adding fucose residues and the synthesis of cutaneous lymphocyte antigen (CLA) carbohydrate determinates (Bengtson et al., 2002; Dudda et al., 2008; Knibbs et al., 1996; Liu et al., 2008; Martinez et al., 2005). Leukocytes, endothelial cells as well as tumor cells are the major FuT7 expressing celltypes, whereas T cells in lymph nodes and resting memory T cells express lower amounts that are enormously enhanced after their activation (Knibbs et al., 1996; Liu et al., 2008; Martinez et al., 2005). FuT7 cell lines carry high avidity E-selectin ligands leading to their slow rolling and homing to centers of inflammation (Dudda et al., 2008). E- and P-selectins are adhesion molecules, whose expression on endothelial cells in the dermis is induced by inflammatory cytokines and highly enhanced during an infection via platelets or endothelial cells' activation. L-selectin is expressed on nearly all leukocytes and bind to L-selectin ligand on endothelial cells. P-selectin is important for leukocyte rolling early at inflammation, whereas L-selectin is essential for rolling of leukocytes that are already attached to vascular cell wall (Bengtson et al., 2002; Martinez et al., 2005). *"Functional E-selectin ligands are expressed on 5-20% of circulating αβ memory T cells, a large percentage of γδ T cells, NK cells and activated T cells after antibody-induced proliferation" (Knibbs et al., 1996)*. Nearly all T cells found in the dermis express E-selectin ligand (Dudda et al., 2008). P-selectin glycoprotein ligand-1 (PSGL-1), expressed on most leukocytes, is the main ligand for P- and E-selectin, whereas E-selectin binds also to E-selectin ligand-1 (ESL-1), but also for L-selectin. PSGL-1 binding to selectins is induced through sLe^x and CLA synthesis mediated by FuT7, whereas sLe^x induction through FuT4 is underpart. FuT7 and FuT4 are seen to can compensate each other in their function of providing selectin ligand. A missense mutation of FuT7 does exist that leads to an inactivation of FuT7 causing no sLe^x generation but although a normal leukocyte rolling on P- and E-selectin occurs due to an increased FuT4 activity (Bengtson et al., 2002; Martinez et al., 2005). *"sLe^x is required to support L-/P-selectin cell rolling on PSGL-1^+FuT7^+ cells, but not on PSGL-1^+FuT4^+ cells" (Martinez et al., 2005)*.

3.3.4.11 Forkhead box P3 (Foxp3)

Foxp3 is a transcription factor that plays a crucial role in *"maintaining peripheral immune tolerance" (Pallandre et al., 2007)* (Fontenot et al., 2003; Hori et al., 2003). Foxp3 has been used as a marker to identify T_{reg}s, although it is debated, if Foxp3 is exclusively expressed on T_{reg}s (Corthay, 2009; Morgan et al., 2005; Wang et al., 2007). However, no alternative marker that is specific for T_{reg}s is known yet. Different observations suggest that $Foxp3^+$ T cells behave like T_{reg}s (Corthay, 2009).

$CD4^+Foxp3^+$ T_{reg}s, which are involved in the regulation of the immune system, are important for establishing and maintaining self-tolerance and for preventing inflammatory and autoimmune diseases in the skin and lungs. Those T_{reg}s are found in many non-lymphoid tissues, especially in the skin, representing 20-40% of all $CD4^+$ T cells in this tissue. Many T_{reg}s in the peripheral blood are carrying cutaneous homing receptors (Dudda et al., 2008; Fontenot et al., 2003; Sather et al., 2007). Skin $CD4^+Foxp3^+$ T_{reg}s generate a normal immune homeostasis in the skin and are probably important in *"preventing cutaneous inflammatory disease" (Sather et al., 2007)* through their cell-mediated immunosuppression leading to cutaneous self-tolerance in mouse models. In absence of $Foxp3^+$ T cells spontaneous autoimmunity can occur (Dudda et al., 2008; Sather et al., 2007).

"The relation of T_{reg}s that constitutively express Foxp3, so-called naive T_{reg} cells, to T_{reg}s that arise in the periphery, induced T_{reg}s, is unclear" (Lee et al., 2005). CD28 as well as ICOS are essential costimulators for the generation and maintenance of T_{reg}s. In $CD28^{-/-}$ or $ICOS^{-/-}$ mice the Foxp3 expression level is markedly decreased (Lee et al., 2005).

T_{reg}s can migrate to lymphoid and also to non-lymphoid tissues that is essential for their induced prevention of aGVHD. Human $CD4^+CD25^+$ T_{reg}s express Foxp3, chemokine (cystein-cystein motif) receptor 4 and 8 (CCR4 and CCR8), whereas the chemokine receptor CCR4 is important to trigger T_{reg}s into skin, lungs and to APCs in secondary lymphoid tissues (Fontenot et al., 2003; Lee et al., 2005; Sather et al., 2007). If a patient, getting a transplant is $CCR4^{-/-}$ the Foxp3 level is much lower after a few days after transplantation compared to a patient with normal CCR4 expression leading to a worse development of tolerance to the transplant (Lee et al., 2005). It has been seen that T_{reg}s that are also $CD25^+$ can lead to improvement of already developed GVHD (Sather et al., 2007).

Foxp3 encodes the transcription factor scurfin (SFN), a winged helix/forkhead protein, which is primarily important in T cell homeostasis by controlling their activation and differentiation (Bennett et al., 2001; Lee et al., 2005; Schubert et al., 2001). When SFN is modified or absent, or when its DNA-binding site is mutated, the T cell homeostasis is destructed leading to an *"early-onset autoimmune disease" (Bennett et al., 2001)*. But SNF can also have the function of a T cell repressor. Different possibilities for this repressor function are discussed: SNF could act as a competitor for positive regulatory elements and disable their binding, SNF could *"actively repress target promoters" (Schubert et al., 2001)*, because other forkhead proteins with this function are known, or it could be a target for the binding of repressive elements (Schubert et al., 2001).

3.4 Diseases

In the following section the most common hematopoietic disorders appearing in our study are explained to overview when HSCT was applied.

3.4.1 Leukemia

Leukemia is a collective name for different types of bone marrow and blood cancer (Piller, 2001). The Greek term leukemia stands for ”white blood”, which indicates that normal blood cell homeostasis have changed towards an augmentation of white blood cells (Abbott, 2006; Lane et al., 2009; McKenzie, 2005). Which cells are involved depends on the type of leukemia. We distinguish primarily between Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL), Chronic Myeloid Leukemia (CML) and Chronic Lymphoblastic Leukemia (CLL) (Chiaretti and Foà, 2009; Mauro and Maziarz, 2006; Stone et al., 2004). The subtypes declare which blood cell types are affected and in which stadium the cells turn into cancer cells (Piller, 2001). But there are some several leukemia cases that cannot be classified into those common subtypes. Also mixed genotypes, like ALL in combination with AML can occur (Chiaretti and Foà, 2009).
Leukemia represents about 2% of all cancer cases (Uzunel, 2003). Approved risk factors for the development of leukemia are high exposure to radiation and chemicals (like benzene). But other unknown factors like the genetic background and genetic disorders, tobacco smoking or a former treatment with cytostatica may also play an important role (Quintás-Cardama and Cortes, 2006; Zeeb and Blettner, 1998).
This malignant disease develops from a disfunction of the bone marrow, where a single cell changes into a highly proliferating cancer cell. Those abnormal cells overgrow the healthy cells and can dispread in the body, if the disease is not treated (Kurzrock et al., 2001; Lane et al., 2009; Onciu, 2009). This usually leads to infections caused by the failure of normal leukocytes. Severe anemia, where the blood fails to carry enough oxygen throughout the body, is caused by the absence of red blood cells. Bruising and hemorrhaging can also occur, which is caused by the absence of platelets (Uzunel, 2003).
Leukemia can be treated in different ways by using specific drugs, cytostatica or by accomplishing HSCT (Barkholt and Ringdén, 2004; Léger and Nevill, 2004). Its aim is a complete remission (CR) which comprises no indication of leukemia cells and a normal blood cell level also after years of treatment (Bettelli et al., 2008; Ikeda et al., 2002). So the patient can turn to its normal life again.

In case of acute leukemia relatively immature blood cells are deformed to cancer cells, whereas more specialized white blood cells are affected in case of chronic leukemia, which has a more slow-going progress compared with the acute form (Pui et al., 2004; Sillaber et al., 2003).

3.4.1.1 Acute Myeloid Leukemia (AML)

The risk of getting AML that *"represents a group of clonal hematopoiectic stem cell disorders" (Stone et al., 2004)* is increasingly higher in older patients (>60 years), although young adults and children can also be affected (McKenzie, 2005; Stone et al., 2004). AML occurs due to the change of one single hematopoietic bone marrow cell leading to a malignant neoplasm through altered proliferation of the myeloid precursor cell, mutations that affect myeloid cell cycle regulation or apoptosis and failure to differentiation. AML cells are more mature than HSC, but have limited self-renewal due to their oncogenic transformation (Lane et al., 2009; McKenzie, 2005; Renneville et al., 2008). AML cells are often called myeloblast cells, representing a cluster of non-functional cells leading to *"loss of normal bone marrow function" (McKenzie, 2005)* through their accumulation (McKenzie, 2005; Stone et al., 2004).

Genetic disorders, such as Down syndrome, and especially chromosome translocations lead to a higher risk of AML development (Renneville et al., 2008; Tenen, 2003). Eight different AML subtypes do exist (M0 to M7) according to the blood cell type affected by leukemia and the nature of leukemia cells (Uzunel, 2003).

Chemotherapy has to be started as soon as AML is diagnosed. The therapy is arranged in two steps: the induction therapy, which stands for the initial treatment with chemotherapy, and the consolidation therapy, where drug treatment for eliminating the remaining cancer cells is accomplished. A CR after five years is only achieved in 40-60% of the cases with this treatment, thus HSCT is very usual in patients with AML (Stone et al., 2004).

3.4.1.2 Acute Lymphoblastic Leukemia (ALL)

ALL is the most common type of leukemia in children (under the age of 15) and occurs more often in children than in adults, but the chance of getting ALL increases again with the age of 45 or older (Pui et al., 2004; Teitell et al., 2009). This leukemia form occurs through abnormal changes in a B or T cell (very rarely in a NK cell) leading to lymphoid neoplasms due to inhibited cell differentiation, abnormal cell proliferation and survival (Onciu, 2009; Teitell et al., 2009). T-ALL, representing about 15% of pediatric and 25% of adult ALL, is less common than B-ALL, but T-ALL is more aggressive. The classification into T- and B-ALL can be subdivided regarding the distinct cell differentiation stage of the neoplastic clone. The precursor B cell stage, which forms precursor lymphoblastic leukemia, can mostly be diagnosed as the cancer cell source of B-ALL, whereas *"pro-T-, pre-T-, cortical and mature T-ALL" (Chiaretti and Foà, 2009)* can appear (Chiaretti and Foà, 2009; Pui et al., 2004; Van Vlierberghe et al., 2008).

Genetic disorders like chromosomal translocations, hyperdiploidy and gene-specific mutations can form the basis of ALL (Pui et al., 2004; Teitell et al., 2009). 2-4% of the children and about 20% of the adult ALL patients have leukemia cells that are carrying the Philadelphia (Ph) chromosome, whereas a chromosome

translocation between chromosome 9 and 22 occurs. The translocation involves the gene ABL1 at chromosome 9 and the gene BCR at chromosome 22, thus the aberrant BCR-ABL1 gene is built making the protooncogene c-ABL oncogenetic leading to alteration of the normal cell growth homeostasis which leads to leukemia (Chiaretti and Foà, 2009; Pui et al., 2004 Quintás-Cardama and Cortes, 2006).
Chemotherapy should be started as soon as possible, whereas children correspond very well to it. 85-90% of pediatric and 40-50% of adult ALL cases have CR and are alive after five years, if they have been treated with chemotherapy (Onciu, 2009). However, relapse can be observed in many patients, depending on the ALL subtype, and those are candidates for HSCT (Pui et al., 2004).

3.4.1.3 Chronic Myeloid Leukemia (CML)

This leukemia form is in contrast to ALL not common in children (<2% of all CML cases) and it is usually diagnosed in individuals older than 30 years, whereas its incidence increases with age. CML that is a *"clonal myeloproliferative disorder of a pluripotent stem cell" (Abramson et al., 1977)* is a rare leukemia form. It represents 14% of all leukemia cases and 20% of adult leukemia incidences (Abramson et al., 1977; Deininger et al., 2000; Quintás-Cardama and Cortes, 2006).
About 95% of the CML patients have leukemia cells that are carrying the Philadelphia (Ph) chromosome and almost half of the remaining patients are carrying the translocated BCR-ABL1 gene as well (Gora-Tybor and Robak, 2008; Mauro and Maziarz, 2006; Quintás-Cardama and Cortes, 2006). The emitted BCR-ABL1 gene that *"exhibits constitutive tyrosine kinase activity" (Sillaber et al., 2003)* leads more frequently to CML than to ALL and very rarely to AML (Sillaber et al., 2003; Kurzrock et al., 1988).
CML is divided into three phases: the chronic phase, where most patients are diagnosed, the accelerated phase and the blast crisis phase. The chronic phase mostly proceeds asymptomatically, because the white blood cells are still working and can combat infections, and diagnose occurs by chance. In the accelerated phase the white blood cell number is abnormal and anemia can be caused by CML, whereas the blast cell number is augmented in the blast crisis phase, thus the red blood cell and platelet number is decreased (Quintás-Cardama and Cortes, 2006; Sillaber et al., 2003).
Leukapheresis is often the first treatment of choice, where the white cells are extracted. This is important, because high levels of white blood cells can downgrate the blood flow to important organs like the brain and the lungs. Drug treatment, mainly with Imatinib mesylate that is a tyrosine kinase inhibitor is very successful in the chronic phase. Remission can be achieved, if the drug is taken continuously. Patients that do not respond to tyrosine kinase inhibitors are treated with IFN-α that has an anti-tumor activity in CML treatment or undergo HSCT (Gora-Tybor and Robak, 2008; Mauro and Maziarz, 2006; Quintás-Cardama and Cortes, 2006).

3.4.1.4 Chronic Lymphoblastic Leukemia (CLL)

CLL is the most common adult leukemia form in the western world representing about 25% of new leukemia incidences and appears primarily in adults older than 50 (Abbott, 2004 and 2006; O'Brien et al., 1995).
95% of CLL are deriving from a malignant B lymphocyte, whereas a T- or NK-CLL type is very uncommon. CLL that is the only form of leukemia that do not arise from the bone marrow can have a very slow progression so that the patients are feeling well and are asymptomatic for a long time, up to years, and no treatment is needed, whereas some patients with CLL have a rapid progression and develop symptoms (Abbott, 2004 and 2006). Different prognostic markers are associated with a more severe form of CLL, like the expression of ζ-chain-associated protein kinase 70 (ZAP70) in B cells, a fast lymphocyte doubling time, loss of Ig heavy chain rearrangements as well as distinct cytogenetic abnormalities (Abbott, 2004; Crespo et al., 2003; Wiester et al., 2003).
A treatment, usually chemotherapy and/or monoclonal antibodies against CLL cells, may be started, when the CLL cells are very increased and the lymph nodes or spleen are enlarged. HSCT is applied very rarely, because chemotherapy leads to a high CR rate (Abbott, 2004 and 2006; O'Brien et al., 1995).

3.4.2 Myeolodysplastic Syndromes (MDS)

MDS is a heterogeneous group of acquired malignant HSC disorders leading to ineffective hematopoiesis, cellular defects and blast cell increase in the bone marrow. MDS is primarily developed in older patients through long term defects of the bone marrow, whereas also activated oncogenes and chromosome aberrations can be involved. However, MDS rarely occurs in younger patients mostly due to applied cytostatica or radiation therapy. MDS affected patients have a higher risk for developing AML. 25% of MDS patients get this leukemia form, whereas younger patients are more prone to it (Anargyrou et al., 2010; Aul et al., 2002).
High-risk MDS patients up to 60 years of age are mostly treated with HSCT, if they are in good conditions, whereas AML-type chemotherapy is also common in young patients (Aul et al., 2002).

3.4.3 Aplastic Anemia

Aplastic anemia is mostly acquired and infrequently inherited, whereas *"children, young adults, and those over 60 years of age" (Keohane, 2004)* are mainly affected (Brodsky and Jones, 2005; Keohane, 2004; Young, 2006).
Aplastic anemia is a rare hematopoietic stem and progenitor cell disorder with destruction of hematopoietic cells in the bone marrow. The hypocellular bone marrow is caused by autoimmune T cell reactions. Some

patients develop a mild form of the autoimmune disease and may not need any therapy, whereas others develop a severe form and are often treated with HSCT. Patients with aplastic anemia are more prone to develop MDS or acute leukemia (Brodsky and Jones, 2005; Keohane, 2004; Young, 2006).

3.4.4 Lymphoma

Malignant lymphomas are a heterogenic group of diseases with neoplastic proliferation of the lymph nodes or lymphatic tissues. Lymphomas are divided into Hodgkin lymphoma, where B cells are affected and non-Hodgkin lymphomas with diverse subtypes that are mostly B cell- and rarely T or NK cell-derived (Hochberg et al., 2009).

4 Materials and methods

4.1 Patient Information

76 patients, who got informed about the project and gave their consult, were included in our study and received HSCT between May 2006 and December 2007 at the Karolinska Institute, Huddinge University Hospital in Stockholm. The median age of the treated patients was 45 (range 10-67). Most of the patients had been diagnosed with leukemia (42 patients; 28 AML and 8 ALL, 5CLL and 1 CML cases), whereas MDS was the second common disease (16 patients). 54 patients had a HLA-matched unrelated donor and 22 patients a HLA-identical relative donor. PBSC were the most common stem cell source (56 patients), but also transplantations with bone marrow stem cells (BMSC) (13 patients) and CBSC (7 patients; 4 got double and 3 got single CBSC) were done. CBSC were used, if no good HLA-matched donor was found. The hematopoietic progenitor cells that were transplanted were selected from the donor samples through their CD34 expression. The median range of $CD34^+$ cells/kg that were transplanted varied between the different stem cell sources. PBSC transplants had a median of 7.8×10^6 $CD34^+$ cells/kg (range 2.1×10^6 - 28.2×10^6), BMSC a median of 2.3×10^6 (range 1.1×10^6 - 7.2×10^6) and CBSC a median of 0.11×10^6 cells/kg (range 0.07×10^6 - 0.72×10^6). About half the patients got RIC with fludarabine (30 mg/m^2/day × 3), busulfan (4 mg/kg/day × 4) and ATG (2 mg/kg/day × 5) administration and the other half got myeloablative conditioning (41 and 35 respectively), whereas busulfan (4 mg/kg/day × 4) and cyclophosphamid (60 mg/kg/day × 2) in combination with TBI (10 Gy) was most commonly used in myeloablative conditioning. Some patients got fractioned TBI (3.6 Gy). 60 out of 76 patients received ATG as prophylaxis for GVHD development. ATG was used in case of unrelated donors as well as part of RIC conditioning due to a higher GVHD risk under these circumstances. GVHD prophylaxis consisted mainly of an administration of cyclosporine A (2 mg/kg/day) and four doses of methotrexate (15 mg/kg/day × 1 + 10 mg/kg/day × 3).

14 patients developed aGVHD grade I, 16 grade II and 5 developed grade III or IV. aGVHD was diagnosed through clinical symptoms, like skin rash, diarrhea or abnormal liver values as defined at the "Consensus conference on aGVHD grading" (Przepiorka et al., 1995). 52% (29 patients) who received PBSC and 45% (6 patients) who received BMSC developed aGVHD, whereas no patient with cord blood as stem cell source developed aGVHD. Graft failure or rejection occurred in 7 patients and 12 patients got relapse. 20 of the patients died, whereof relapse (9 patients) was the main cause of death. The patient characteristics are listed up and described in more detail in table 1. Figure 2 graphically shows the frequency of aGVHD development in our study.

	Number of patients
HSCT treated patients	76
Age, median (range) years	45 (10-67)
Diagnosis	
Aplastic anemia	4
Acute leukemia	36
Chronic leukemia	6
Lymphoma	3
Multiple myeloma	1
Solid tumour	2
MDS/MPS	16
Metabolic diseases	6
Myelofibrosis	1
Immunocytomegali	1
Donor	
HLA-identical (sibling, parent)	22
HLA-matched unrelated donor	54
Stem cell source	
Peripheral blood stem cells	56
Bone Marrow	13
Cord Blood	7
Conditioning	
RIC	41
Myeloablative	35

	Number of patients
Antithymocyte globulins	60
GVHD prophylaxis	
MTX+CsA	58
Pred+CsA	6
FK+Rapa	12
Acute GVHD	
no	39
grade I	14
grade II	16
grade III/IV	5
CMV reactivation	36
Bacteremia	15
Graft failure/rejection	7
Relapse	12
Dead	20
Relapse	9
Fungal infection	3
Pneumonia	3
EBV-lymphoma	2
GVHD	1
Liver disease	1
Other	1

Table 1: Patient information
MDS indicates myelodysplastic syndromes; MPS, mucopolysaccharidoses; RIC, reduced intensity conditioning; HLA, human leukocyte antigen; MTX, methotrexate; CsA, cyclosporine A; Pred, prednisolone; FK, tacrolimus; Rapa, rapamycin; CMV, Cytomegalovirus; EBV, Ebstein Barr Virus.

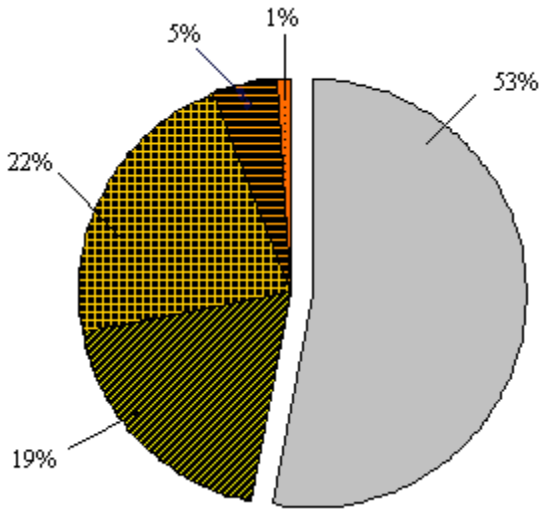


Figure 2: aGVHD cases
Grey: no aGVHD; diagonally striped: aGVHD grade I; checkered: aGVHD grade II; horizontally striped: aGVHD grade III; dotted: aGVHD grade IV

4.2 Preface

In our study RQ-PCR and ELISA were the methods of choice. RQ-PCR made it possible to measure the cytokines' mRNA levels in $CD4^+$ and $CD8^+$ T cells. We were analyzing the mRNAs of IFN-γ, IL-10, IL-18, ICOS, Foxp3, FuT7 and B7-int, whereas we measured the cytokine protein plasma levels of IFN-γ, IL-10, IL-18, IL-12, IL-17 and IL-23 with ELISA. IFN-γ, IL-10 and IL-18 were analyzed both with RQ-PCR and with ELISA.

ICOS, Foxp3, FuT7 and B7-int are membrane bound and therefore could not be measured in the plasma. Whereas IL-12 is mainly distributed by phagocytic cells, B cells and dendritic cells, it could not be detectable in T cells' mRNA. IL-23 is only secreted at low amounts of T cells and was therefore not measured via RQ-PCR. IL-17 is however mainly distributed by T_H17 cells and because we analyzed the gene expression levels in $CD4^+$ and $CD8^+$ T cells, only the plasma protein level of IL-17 was studied.

The following steps that are schematiclally listed upp in figure 3 were done to analyze all patients' blood samples.

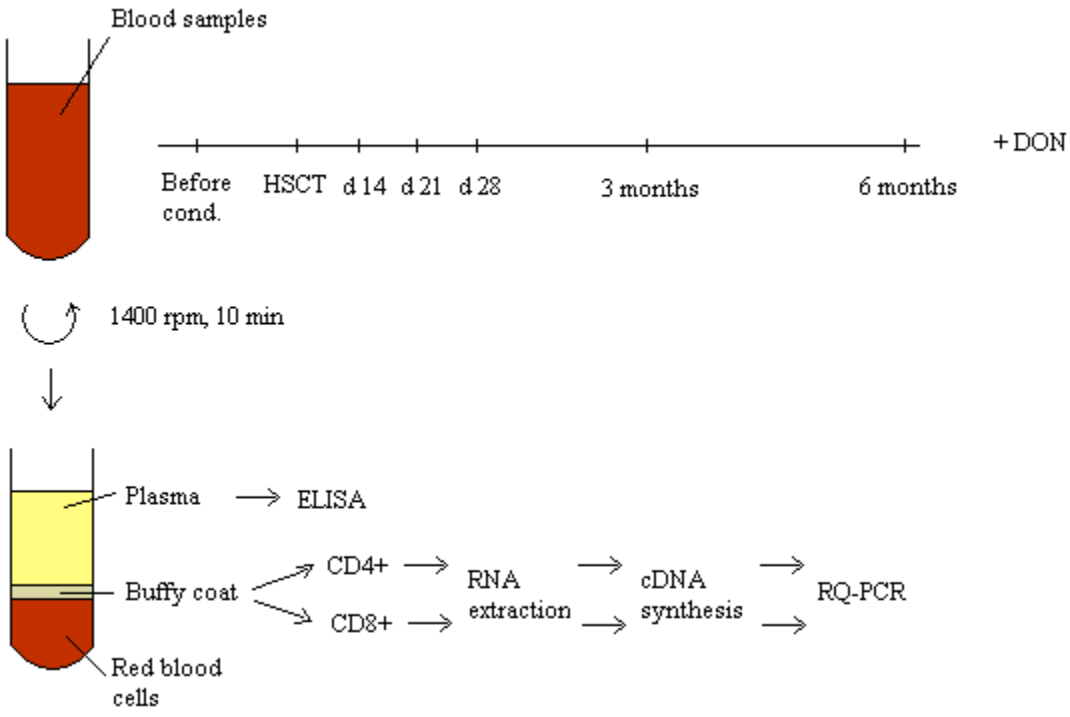


Figure 3: Surfey of the steps
Cond., conditioning; d, days after HSCT; ELISA, enzyme-linked immunosorbent assay; HSCT, hematopoietic stem cell transplantation; rpm, rounds per minute; RQ-PCR, real time-quantitative polymerase chain reaction

4.3 Samples

The patient samples consisted of 5-10 ml of peripheral blood and were collected at 7 different timepoint occasions: before conditioning, at the day of HSCT, 14, 21, 28 days, 3 and 6 months after HSCT. A total of 936 patient samples ($CD4^+$ and $CD8^+$ T cells) were analyzed. The donor samples consisted also of 5-10 ml of peripheral blood or 1-5 ml of bone marrow. Cord blood samples were not analyzed, because the cell amount was low and the whole isolated blood was needed for the transplantation. A total of 168 donor samples were analyzed, whereas in some cases only donor and no patient samples were available.

4.4 Plasma and T cell Isolation

The plasma samples were obtained from peripheral blood samples collected from the patients, as well as from peripheral blood or bone marrow donor samples. The samples were centrifuged (1400 rpm, 10 min, 4˚C) to get different phases. The plasma (upper phase) was stored at -20 °C and then used for the ELISA assay.
The cells in the buffy coat (including most of the white blood cells, therefore also the interesting T cells) were collected and beaded with immunomagnetic beads specific against $CD4^+$ T cells. The $CD4^+$ T cells (bound to the beads) were separated with a magnet and the remaining coat sample was beaded again with beads specific against $CD8^+$ T cells and separated. The two different T cell populations were purified by washing with phosphate buffered saline (PBS) to get rid of the unbound cells.

4.5 RNA Extraction

The RNA extraction was operated with the *Qiagen RNeasy Mini Kit* (Qiagen, Hilden, Germany).
The RLT lysis buffer for disruption and homogenization and β-mercaptoethanol (14,3 M) was added to the $CD4^+$ and $CD8^+$ T cells, respectively to break up the cell walls and plasma membranes (disruption) to release the cell contents, whereas the RNA was of interest. The RLT buffer contained guanidine thiocyanate to inactivate RNases. β -mercaptoethanol denatured proteins through its capacity to cleave disulfide bonds. Homogenization was important to lower the viscosity of the cell lysate so that the RNA could bind more efficiently to the RNeasy spin column (in the next step). The RNA got stabilized and the cell lysates were stored at -75 °C.
The thawed $CD4^+$ and $CD8^+$ T cell lysats had to get rid of the magnetic beads by using a magnet so that the beads could not influence the RNA extraction process. 70% ethanol was added leading to precipitation of DNA and RNA. The RNA could bind to the specific ion exchange column and the bulk of the DNA was

washed through a filter. Some DNA contaminations always occurred. RW1 and RPE buffers containing ethanol were used as washing buffers for a more efficient RNA binding to the filter membrane. RNase-free water was added for washing the purified RNA, which is water soluble, from the membrane.
The RNA concentration was measured with a spectrophotometer at a 1:10 dilution. The 260nm/280nm yield delivered a possible protein contamination of the sample. There was no contamination, if the yield lay between 1,7 – 2,0 and not above. RNA absorbed the light at 260nm. The samples were nearly always not contaminated. The RNA samples were diluted to an end concentration of not more than 50 ng/ml with an end volume of 30 µl, because the RQ-PCR signal would not correlate with the RNA yield (respectively cDNA) at to high RNA amounts so that primers and probes would not be able to bind all specific cDNA. The primer and probe concentration could not be increased neither, because it would result in a more unspecific binding to the RNA, adulterating the measuring.

4.6 cDNA Synthesis

cDNA was built form the RNA for the RQ-PCR. It included 1× first strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 105 µg/ml pdN_6 (random hexamer primer; Pharmacia Biosciences, Uppsala, Sweden), 1 mM DTT (dithiothreidol; Promega, Madison, WI, USA) and 1 mM of each dNTP (deoxyribonucleotide; Pharmacia Biosciences). 4.8 units/µl M-MLV RT (reverse transcriptase; Invitrogen, Paisley, Scotland) and 0.48 units/µl RNasin (Promega) were added immediately before the cDNA synthesis was started.
DTT was used for reducing disulfide bonds and as a stabilizer for the Taq polymerase (reverse transcriptase).
The RNA samples had to be heated at 68 °C for 10 min. before the cDNA mix (21 µl for each sample) was added to open secondary and tertiary structures of the RNA so that the primers could bind. The samples were incubated at 37 °C for 1½ hours. RT and the RNasin are most active at this temperature. When RNA was bound to cDNA, it was broken down by RT, whereas RNasin stabilized the RNA in its unbound form. After incubation time the samples were heated at 68 °C for 2 min. to inactivate the enzymes. The samples were diluted with 80 µl RNase-free water and stored at -20 °C.

4.7 Real time Quantitative Polymerase Chain Reaction (RQ-PCR)

RQ-PCR has a big advantage over standard PCR, which just delivers cDNA information on qualitative analysis. Furthermore a post-PCR analysis (gel-electrophoresis) is required in the standard PCR to make this statement. The RQ-PCR is a quantitative method, where the PCR products are accumulated in real time, the

PCR products are doubled in every cycle. The detection of the product is done through measuring the fluorescence signals generated during the PCR process. The fluorescence signal can be produced in different ways through different methods (Uzunel, 2003).

We used the TaqMan-probes, where two fluorochromes, a reporter fluorochrome at the 5' end and a quencher fluorochrome at the 3' end were bound. If the probe bound to the DNA, the reporter and quencher were in close distance to each other and therefore the quencher could silence the fluorescence signal, which was emitted from the reporter dye. This occurred because the quencher dye had lower fluorescence energy than the reporter dye and it took up the free, from the reporter dye emitted electrons leading to no fluorescence signal in the reporter dye wavelength. Only a quencher specific fluorescence signal appeared.

During polymerization the probe was displaced by the Taq-polymerase and hydrolyzed by the 5' to 3' exonuclease activity of this enzyme. The reporter and quencher were more distant to each other and therefore lost the quencher its function. Thus a reporter fluorescence signal could be measured. The intensity of the signal was doubled in every cycle in the logarithmic phase. The signal formation and polymerization during the RQ-PCR run is shown in figure 4.

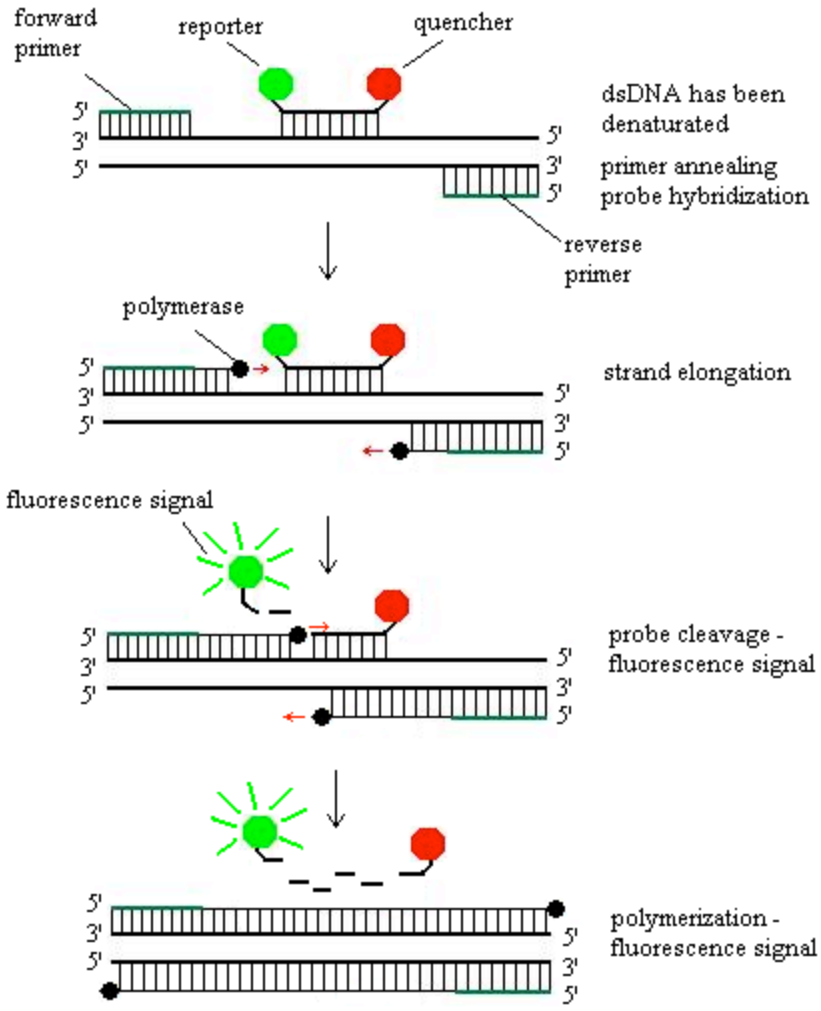


Figure 4: RQ-PCR with Taqman-probe
The fluorescence signal of the reporter dye can be measured, if the quencher is more distant to the reporter, thus is no longer able to absorb the reporter's fluorescence signal.

The total cDNA (corresponding to the RNA) amount could be measured by using a standard curve with known cDNA concentrations. The concentration correlated with the fluorescence intensity and therefore with the threshold cycle (C_t) value (see below). We were instead only interested in a relative quantification, where the RNA amount of one gene was seen as its correlation to another. Therefore no standards were used. We used the G6PD (Glucose-6-phosphate dehydrogenase) gene as internal reference gene that is part of the pentose phosphate pathway and is relative constantly expressed in T cells providing to see if a higher signal in different genes was due to more cells (resulting also in a higher G6PD signal) or to a higher RNA amount (G6PD signal was not altered).

The C_t was defined as the cycle where the fluorescence signal could be measured over a distinct threshold and could therefore be distinguished from the background. If the C_t was low, the mRNA (cDNA) concentration was high.

FAM (6-carboxy fluorescein; Applied Biosystems, Foster City, CA, USA) was used as reporter and TAMRA (6-carboxy-tetramethyl rhodamine; Applied Biosystems) was used as quencher dye. FAM had its emission maximum at 520 nm and TAMRA its excitation maximum at 555 nm and its emission maximum at 576 nm. TAMRA was able to absorb the emission light of FAM, because it did not only absorb light at the wavelength of 555 nm and FAM emitted light up to 570 nm.

The ABI 7000 Sequence Detection System (Applied Biosystems) was used for RQ-PCR running.

The PCR mix included 1×TaqMan Universal PCR Master Mix (Applied Biosystems, containing dNTPs with deoxyuridine-triphosphate (dUTP), $MgCl_2$, AmpliTaq Gold® DNA polymerase, AmpErase® uracil-DNA-glycosylase (UNG) and 300 nM of gene specific primers (Applied Biosystems) and 200 nM of probes. UNG was used for preventing false positives through its capacity to remove uracil from PCR contaminating products, such as from probe-bound RNA.

22.5 µl mix and 2.5 µl samples were added in each well and centrifuged (1400 rpm; 45 sec) to get rid of bubbles at the bottom that could influence the RQ-PCR.

The following primer and probe sequences for Foxp3, IFN-γ, IL-10, IL-18, ICOS, FuT7, B7-int and G6PD were used:

Foxp3 forward primer: 5'-AACAGCACATTCCCAGAGTTCCT-3'

Foxp3 reverse primer: 5'-CATTGAGTGTCCGCTGCTTCT-3'

Foxp3 probe: 5'-FAM-CCTTTCACCTACGCCACGCTCATCC-TAMRA-3'

IFN-γ forward primer: 5'-GAAAAGCTGACTAATTATTCGGTAACTG-3'

IFN-γ reverse primer: 5'-GTTCAGCCATCACTTGGATGAG-3'

IFN-γ probe: 5'-FAM-TTGAATGTCCAACGCAAAGCAATACATGA-TAMRA-3'

IL-10 forward primer: 5'-GAGGCTACGGCGCTGTCAT-3'

IL-10 reverse primer: 5'-AGATGCCTTTCTCTTGGAGCTTATT-3'

IL-10 probe: 5'-FAM-CAAGAGCAAGGCCGTGGAGCAGG-TAMRA-3'

IL-18 forward primer: 5'-GACCAAGGAAATCGGCCTCTA-3'

IL-18 reverse primer: 5'-ACCTCTAGGCTGGCTATCTTTATACATAC-3'

IL-18 probe: 5'-FAM-ACTGATTCTGACTGTAGAGATAATGCACC-TAMRA-3'

ICOS forward primer: 5'-CCATAGGATGTGCAGCCTTTG-3'

ICOS reverse primer: 5'-GGTCGTGCACACTGGATGAA-3'

ICOS probe: 5'-FAM-CATTTTGGGATGCATACTTATTTGTTGGCTTACA-TAMRA-3'

FuT7 forward primer: 5'-CGGCTGCCGTGATTTGAG-3'

FuT7 reverse primer: 5'-GGCCGTGCCCAGCAT-3'

FuT7 probe: 5'-FAM-TTGGCTGACTGATCCTGGGAGACTGTG-TAMRA-3'

B7-int forward primer: 5'-CAGCACAGAGTTTGACTACCCTTCT-3'

B7-int reverse primer: 5'-GGCACTGGTGACAGCAAAGA-3'

B7-int probe: 5'-FAM-CCTCTCTGCAGCAAATATCCAGCC-TAMRA-3'

G6PD forward primer: 5'-TGCCCCCGACCGTCTAC-3'

G6PD reverse primer: 5'-ATGCGGTTCCAGCCTATCTG-3'

G6PD probe: 5'-FAM-ACTCGTGAATGTTCTTGGTGACGGCC-TAMRA-3'

The RQ-PCR was run according to the scheme (Table 3). All samples were run in duplicates using 96-well plates. The threshold (between 0.15 and 0.20) was adjusted manually, which allowed a precise tuning after a RQ-PCR run. The baseline was set manually between the first 3 cycles and about 3 cycles before the first exponential increase crossing the threshold line.

PCR cycle			
Stage 1	Stage 2	Stage 3 step1	Stage 3 step2
1x 50°C 2 min	1x 95°C 10 min	40x 95°C 15 sec	40x 60°C 1 min
UNG Deactivation	Polymerase Activation	Denaturation	Annealing Elongation

Table 3: PCR cycle
Stage 1 and 2 were only perfomed at the beginning of the RQ-PCR run. Step 1 and 2 of stage 3 were run alternating for 40 times.

4.8 Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA was the method of choice to measure the protein expression level. The proteins, in which we were interested, were the cytokines that were probably relevant in GVHD: IL-10, IL-12, IL-17, IL-18, IL-23 and IFN-γ. Figure 5 shows a schematic overview of the different ELISA steps.

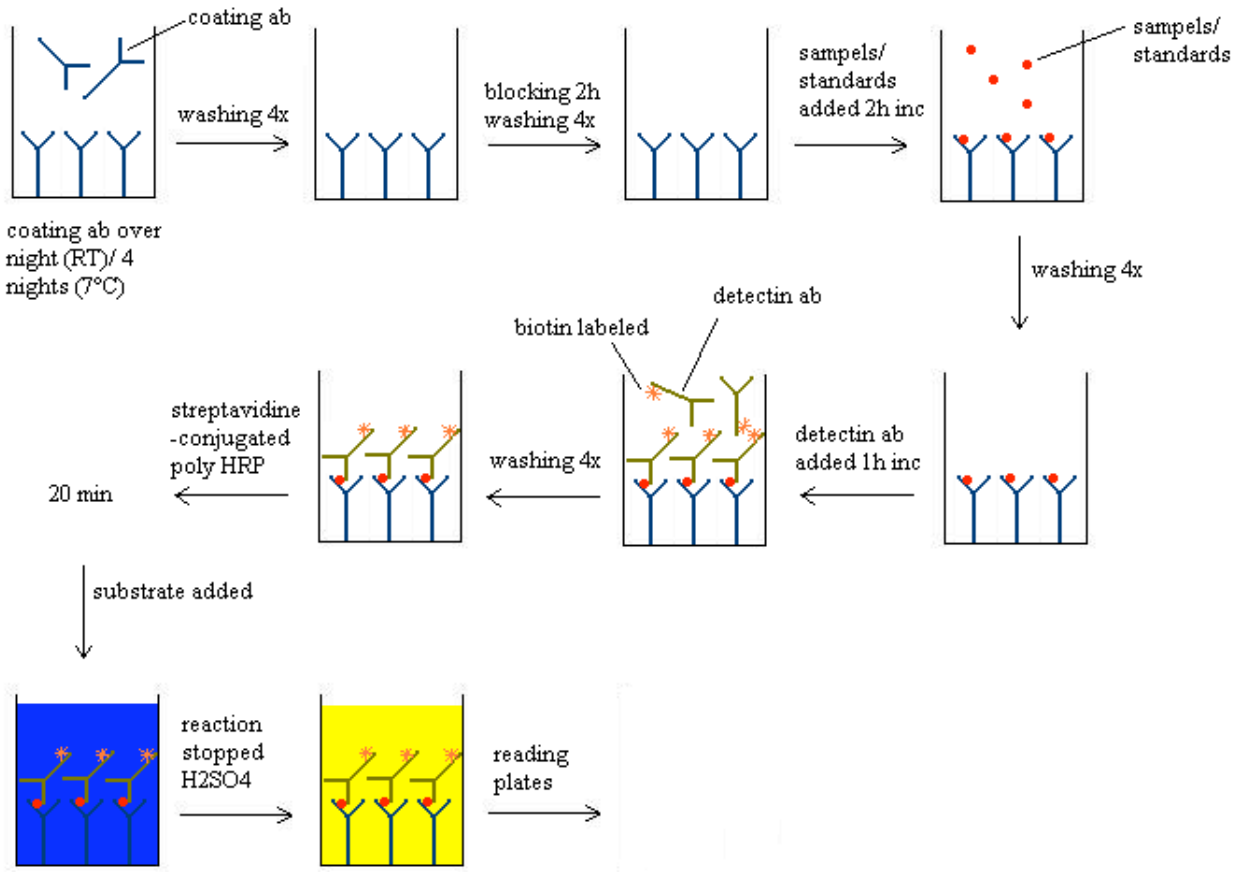


Figure 5: Sandwich ELISA
The wells turn blue after addition of the substrate, if enough biotin-labeled detection antibodies can bind (positive samples). If the reaction gets stopped with H2SO4, the wells turn into yellow. For description see text.
Ab, antibody; h, hours; HRP, horseradish peroxidase; inc, incubation; RT, room temprature

The ELISAs were put up with the help of the "ELISA development guide" provided by R&D Systems (Minneapolis, MN, USA), using flat-bottom polystyrene 96 well half-area enzyme immunoassay plates (Costar, Cambridge, MA, USA), thereby allowing an addition of 50 µl per well in each step instead of 100 µl as in the manual. Coating was performed with a cytokine specific antibody overnight at room temperature (R&D Systems coating antibodies for IL-10, IL-12 and IL-17; MBL, Woburn, MA, USA coating antibody for IL-18; eBioscience, San Diego, CA, USA coating antibody for IL-23) and during four nights at +7 °C (IFN-γ, R&D Systems), with the antibodies diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plates were washed four times in a 0.15 M sodium chloride (NaCl) 0.05% Tween-20 buffer (after each step) to get rid of the unbound antibodies. Blocking was performed during two hours in PBS buffer containing 1% bovine serum albumin (BSA) to avoid unspecific bindings. Samples, recombinant human cytokines (R&D Systems for IL-10, IL-12, IL-17, IL-23, IFN-γ; MBL for IL-18) for standard curves, detection antibodies and streptavidine-conjugated poly horseradish peroxidase (HRP; Sanquin Reagents, Amsterdam, The Netherlands) were diluted in 1% BSA and 0.05% tween in PBS (IL-10, IL-17, IL-18), and high performance ELISA (HPE) dilution buffer (Sanquin Reagents) diluted 1:8 in sterile water (IL-12, IL-23, IFN-γ). The detection antibodies were diluted in 1% BSA and 0.05% tween in PBS or HPE dilution buffer, respectively containing 2% goat serum. After blocking and thoroughly washing the samples, different standard

concentrations and a blank (50 µl/well) were added in duplicates. The incubation time was 2 hours so that the samples and standards could bind to the immobilized antibodies. After another washing step for getting rid of the unbound analytes a biotin conjugated detection antibody (R&D Systems detection antibodies for IL-10, IL-12, IL-17 and IFN-γ; MBL detection antibody for IL-18; eBioscience detection antibody for IL-23) that could bind to another analyte-epitope was added and incubated for 1 hour. The streptavidine-conjugated poly-HRP (diluted 1:10.000) detection agent was added after washing to bind to the biotin-labeled detection antibody (20 minutes incubation). In the final step a substrate solution containing tetramethylbenzidine dihydrochloride - 1 tablet/10 ml (SIGMA, Saint Louis, MO, USA) and hydrogen peroxide (1:10.000) in 0.05 M phosphate-citrate buffer (pH 5.0) was added and the reaction was stopped by the addition of 50 µl 1M sulfuric acid (H_2SO_4), when the product got colored bluish. The reaction was stopped after not more than 20 minutes even though the substrate did not turn blue to avoid false positives.

The concentrations of the coating, detection antibodies, sample dilutions and standard curves were listed up in table 4.

	Coating-ab (µg/ml)	Standard curve (pg/ml)	Detection-ab (ng/ml) 2% goat serum	Sample dilution
IL-10	1	2,1 - 1500	50	01:02
IL-12p70 (IL-12)	4	8,2 - 6000	50	01:02
IL-12p40 (IL-23)	4	8,2 - 6000	500	01:02
IL-17	4	4,1 - 3000	50	01:02
IL-18	2	1,4 - 1000	125	1:30 1:90
IL-23	2	8,2 - 6000	500	01:02
IFN-γ	1	8,2 - 6000	50	01:02

Table 4: ELISA details
Antibody concentrations, standard curves and sample dilutions; ab, antibody

The recombinant human antibodies were used for standard curves. They were diluted threefold in order to see the lower and the linear part of the sigmoidal curve. All plates were read with the VMax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm, using 550 nm as reference wavelength, and the results were analyzed with help of Softmax Pro 3.0 (Molecular Devices). The detection level of the distinct cytokines was set at the initiation of the linear phase of the sigmoidal curve of each standard curve. The sensitivity in detection was in the range of 10-1000 pg/ml for IL-10, 20-1000 pg/ml for IL-12, 50-1000 pg/ml for IL-17, 5-1000 pg/ml for IL-18, 200-2000 pg/ml for IL-23 and 70-5000 pg/ml for IFN-γ. The samples, which concentrations did not exceed the lowest detection level were set as half of that concentration (5 pg/ml for IL-10, 10 pg/ml for IL-12 and so one).

4.9 Statistical Analysis

Survival and incidence curves were generated using the Kaplan-Meier method. For univariate and multivariate analysis, the Cox proportional hazards regression model was used. Mann-Whitney U test and Fisher's exact tests were used to evaluate differences between groups. Analyses were performed using Statistica software (StatSoft, Tulsa, OK, USA).

5 Results

5.1 RQ-PCR Results

5.1.1 Overall Gene Expression Levels at Different Timepoint Occasions

The different gene expression levels in $CD4^+$ and $CD8^+$ T cells of donors and all patients were compared, not distinguishing patients with and without GVHD, to see an overall trend of the gene expression levels' expansions from the time period of conditioning to 6 months after transplantation, as shown in figure 6a-g. This may elucidate which cytokine productions got more influenced by conditioning as well as HSCT and how fast, if at all, they reached their pre-conditioning states again.

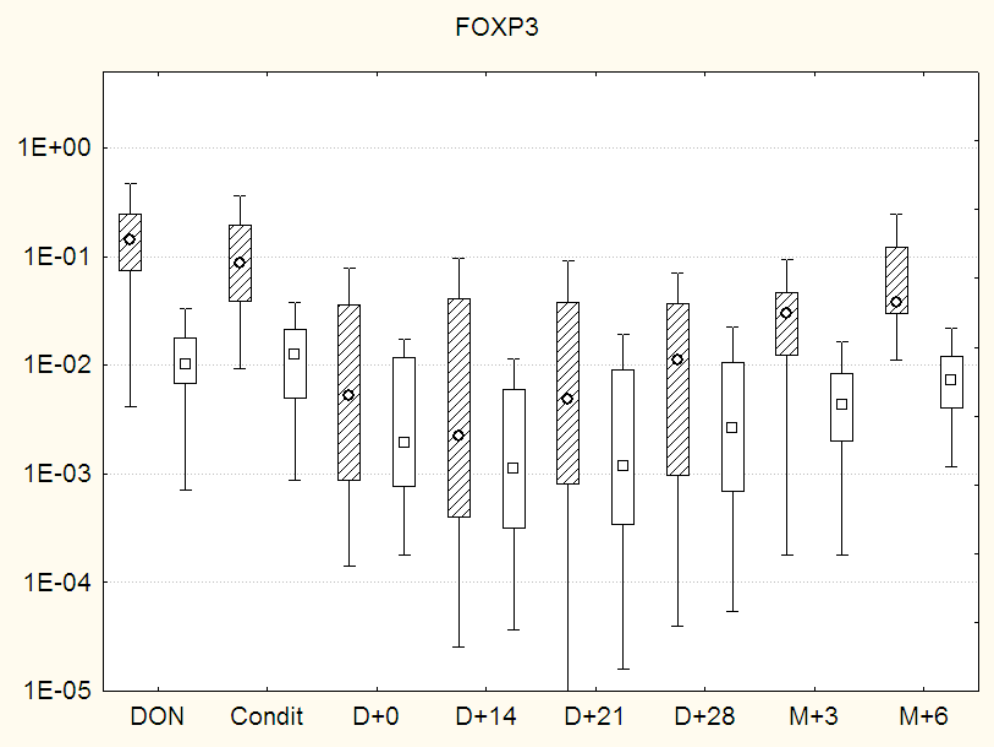


Figure 6a: Foxp3 gene expression in $CD4^+$ and $CD8^+$ T cells
black striped - $CD4^+$ T cells; white - $CD8^+$ T cells; DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

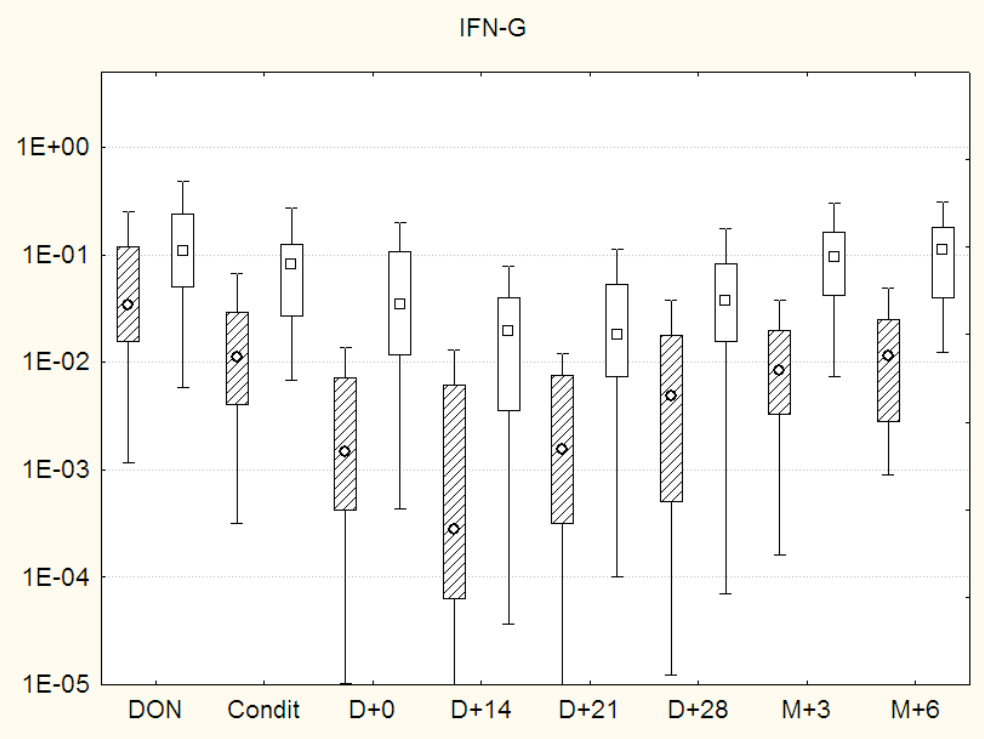


Figure 6b: IFN-γ gene expression in CD4⁺ and CD8⁺ T cells
black striped - CD4⁺ T cells; white - CD8⁺ T cells; DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

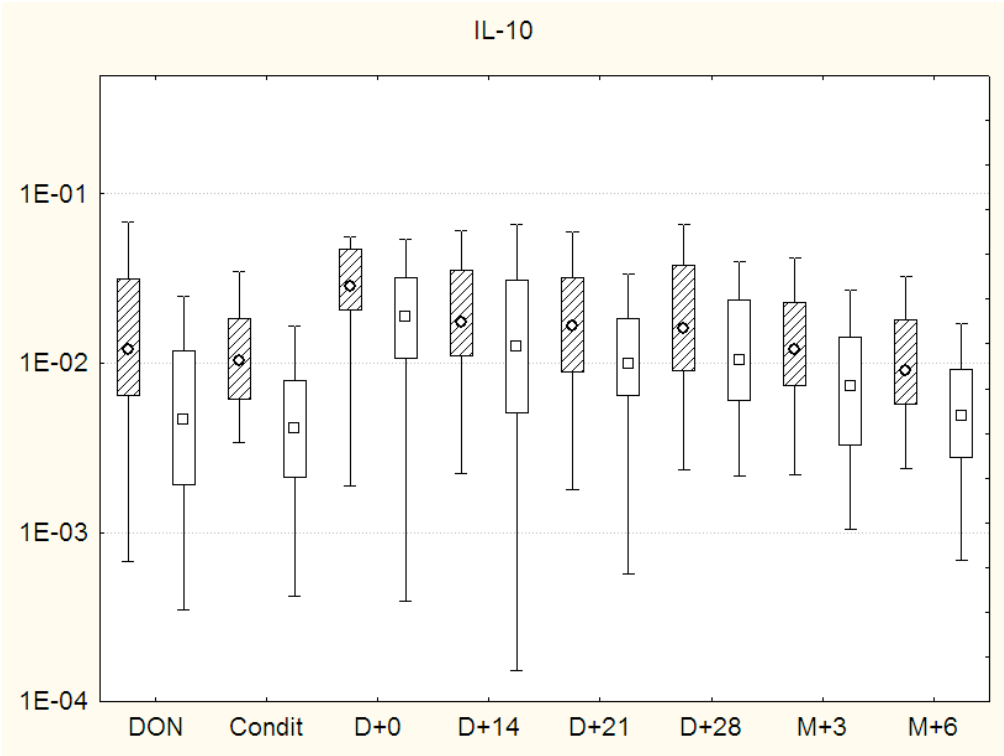


Figure 6c: IL-10 gene expression in CD4⁺ and CD8⁺ T cells
black striped - CD4⁺ T cells; white - CD8⁺ T cells; DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

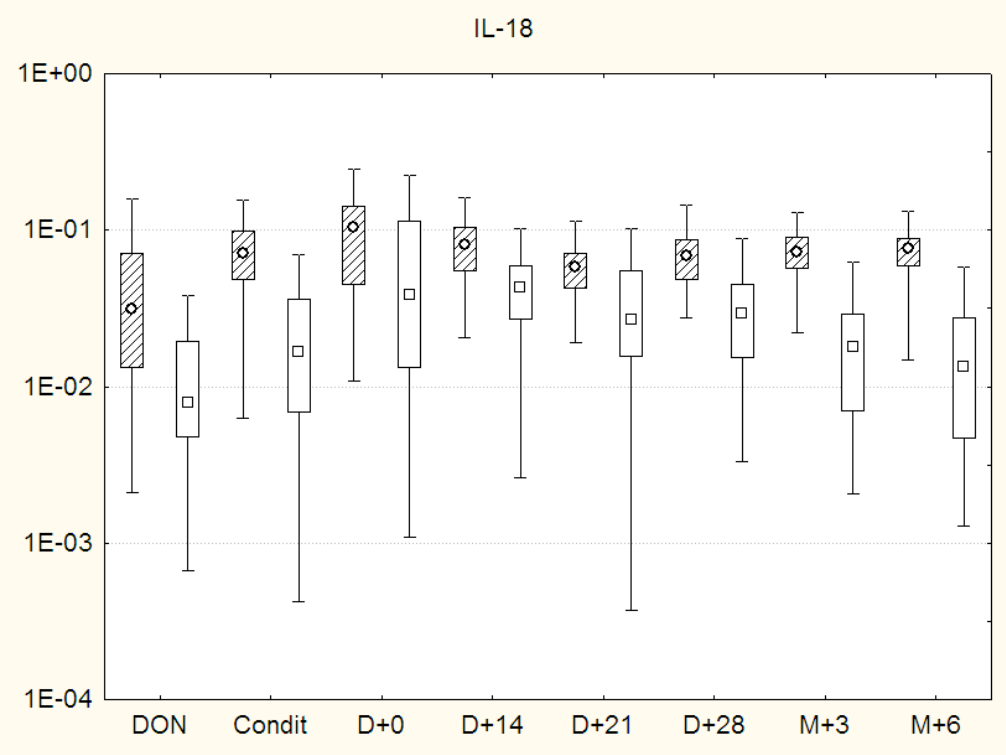


Figure 6d: IL-18 gene expression in CD4⁺ and CD8⁺ T cells
black striped - CD4⁺ T cells; white - CD8⁺ T cells; DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

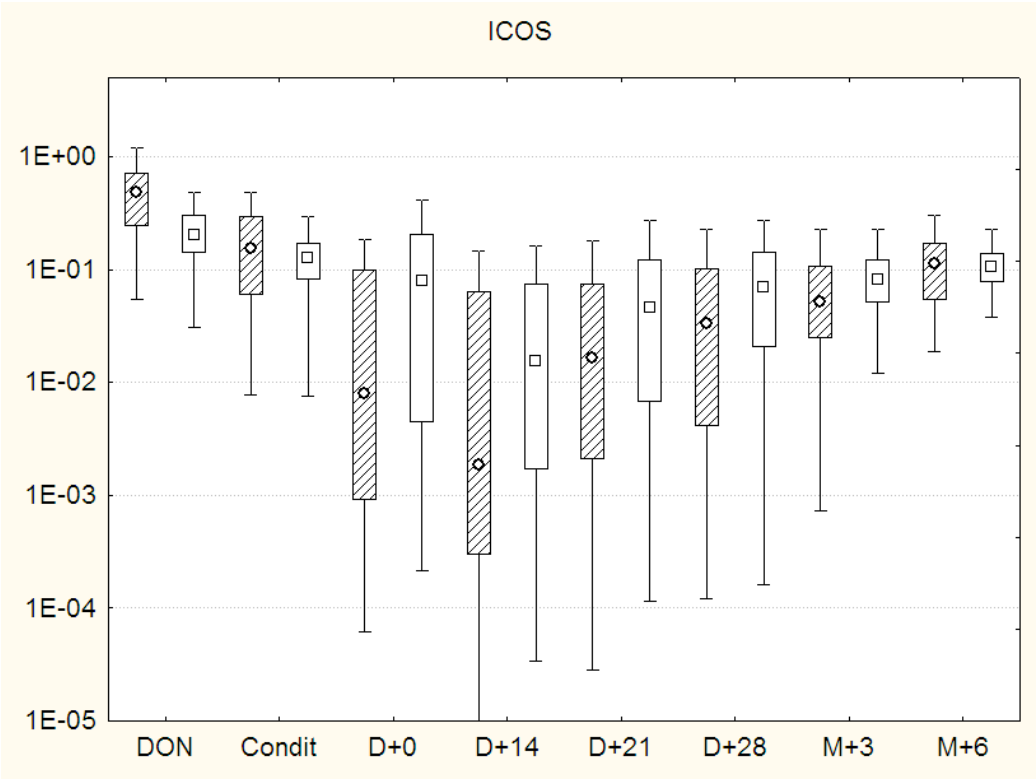


Figure 6e: ICOS gene expression in CD4⁺ and CD8⁺ T cells
black striped - CD4⁺ T cells; white - CD8⁺ T cells; DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

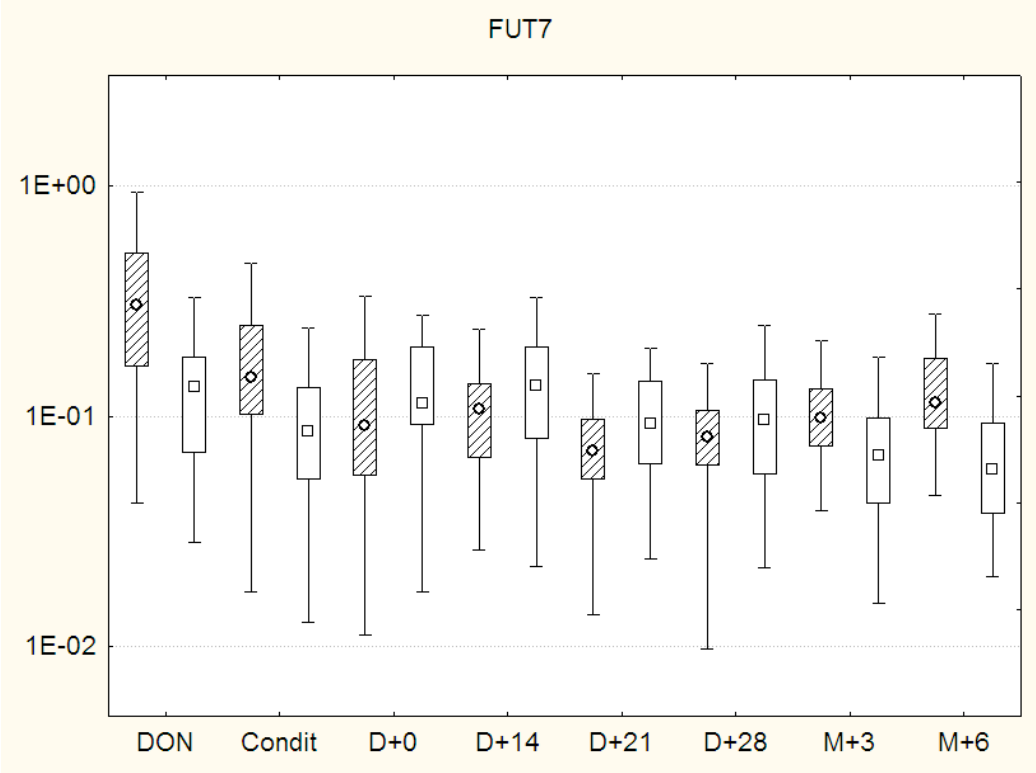


Figure 6f: FuT7 gene expression in CD4⁺ and CD8⁺ T cells
black striped - CD4⁺ T cells; white - CD8⁺ T cells; DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

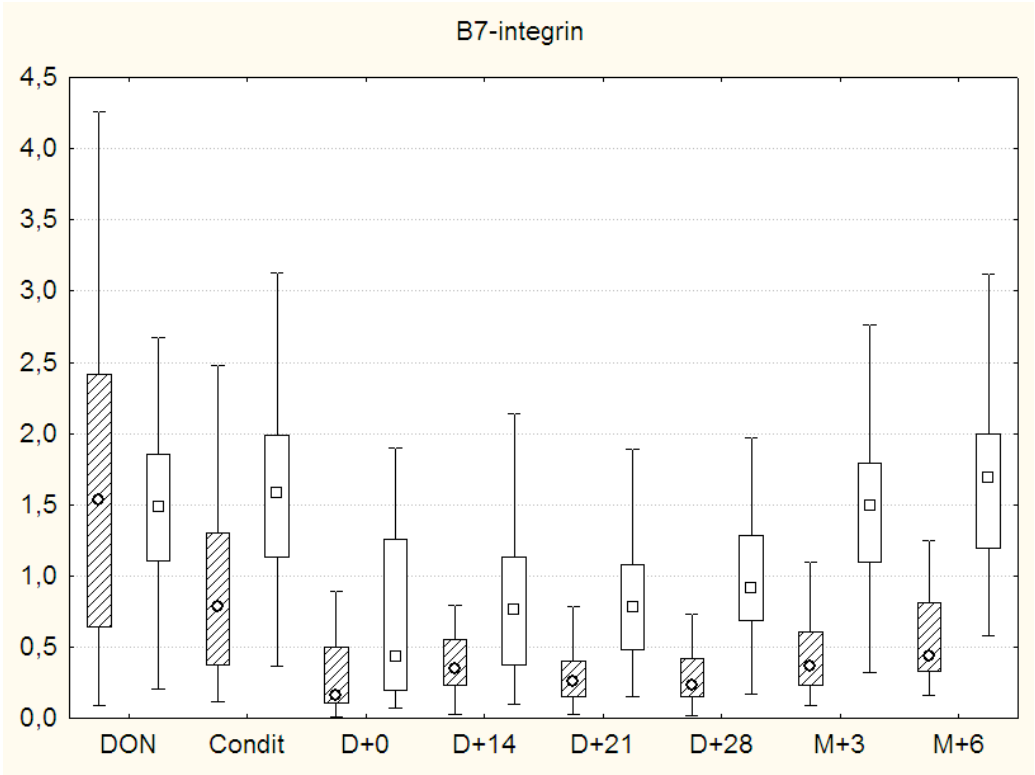


Figure 6g: B7-int gene expression in CD4⁺ and CD8⁺ T cells
black striped - CD4⁺ T cells; white - CD8⁺ T cells; DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

Comparing the donors' gene expression levels with the patients' pre-conditioning gene expressions in $CD4^+$ and $CD8^+$ T cells, respectively, no significant differences of Foxp3, IFN-γ, IL-10, IL-18, ICOS and FuT7 expression levels were seen (figure 6a-f). But the mRNA level of B7-int in $CD4^+$ T cells was lower in pre-conditioning patients' than in donors' samples, although not significantly (figure 6g).

Looking at the donors' or patients' pre-conditioning mRNA levels of Foxp3, IL-10, IL-18 and FuT7, genes were stronger expressed in $CD4^+$ T cells than in $CD8^+$ T cells, whereas IFN-γ was more expressed in $CD8^+$ T cell and ICOS had comparable mRNA levels in both T cell types. We measured equal B7-int gene expression levels in donors' $CD4^+$ T cells and $CD8^+$ T cells, but a lower B7-int expression in patients' $CD4^+$ T cells than in $CD8^+$ T cells at pre-conditioning state that was comparable with the donors' B7-int level in $CD8^+$ T cells.

Foxp3 and IFN-γ showed both a downregulation of their mRNA levels after conditioning, but the levels increased again 14 days after transplantation. Foxp3 reached a normal, pre-conditioning level after 6 months and IFN-γ after 3 months. The conditioning regime did not shift the expression of Foxp3 or IFN-γ from more expressing $CD4^+$ T cells to more expressing $CD8^+$ T cells or vice versa. Foxp3 had always higher mRNA levels in $CD4^+$ T cells and IFN-γ in $CD8^+$ T cells.

The ICOS gene expression level strongly decreased in $CD4^+$ T cells, but only slightly in $CD8^+$ T cells after conditioning, so that not both T cell types were expressing likely the same amount of ICOS any more. The gene expression level of ICOS increased 14 days after transplantation and reached a normal, pre-conditioning level after 3 to 6 months, whereas both T cell types were expressing the same ICOS mRNA amount again.

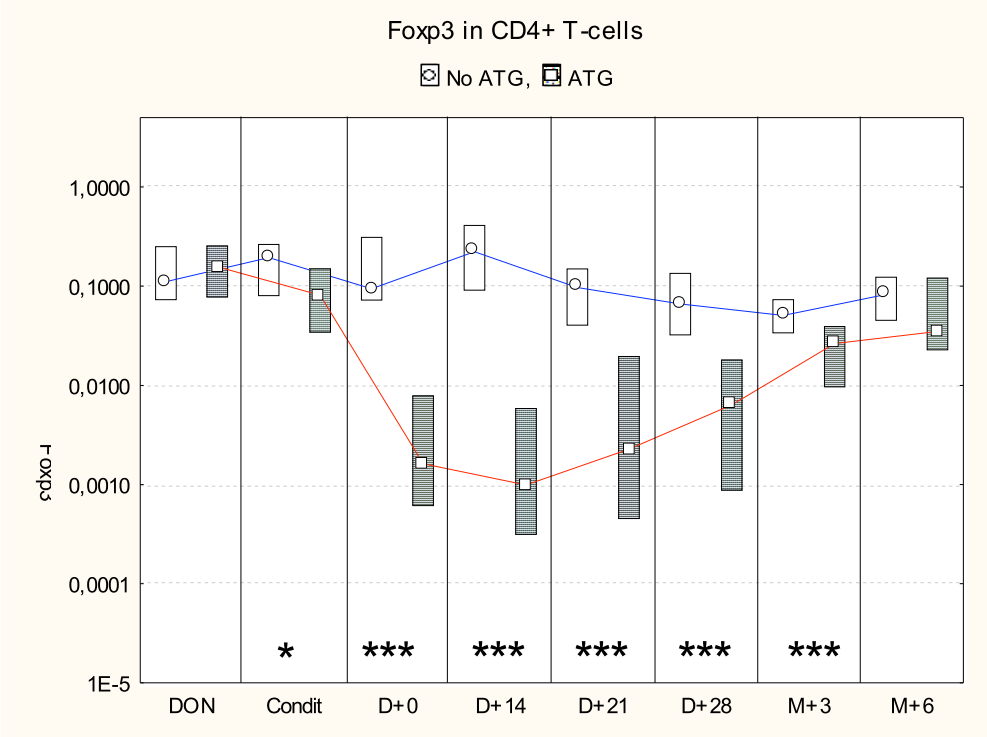
The overall gene expression level of FuT7 was in principle the same, whereby $CD8^+$ T cells were producing more FuT7 than $CD4^+$ T cells after conditioning, thus the T cell types changed their expression levels. 3 months after transplantation $CD4^+$ T cells were producing more FuT7 than $CD8^+$ T cells again, as under pre-conditioning circumstances.

The gene expressions of B7-int was very decreased after conditioning, whereas $CD8^+$ T cells were expressing more B7-int than $CD4^+$ T cells, like in the patients' pre-conditioning state. The B7-int mRNA level slightly increased after 3 months and was comparable with the pre-conditioning state after 6 months.

IL-10 and IL-18 were the only genes measured in this study that increased after conditioning, whereas their gene expression was always stronger in $CD4^+$ than in $CD8^+$ T cells. IL-10 had a strong and IL-18 only a weak increase. Both IL-10 and IL-18 reached a pre-conditioning gene expression state 3 months after transplantation.

5.1.2 Gene Expression Levels in T Cells - with and without ATG

ATG was used for depleting T cells after HSCT, thus reducing the risk of aGVHD (see page 15). We were therefore interested if there were any differences of the cytokine gene expression levels between patients, who received ATG and patients, who did not, thus may explaining the diverse outcome of aGVHD development.



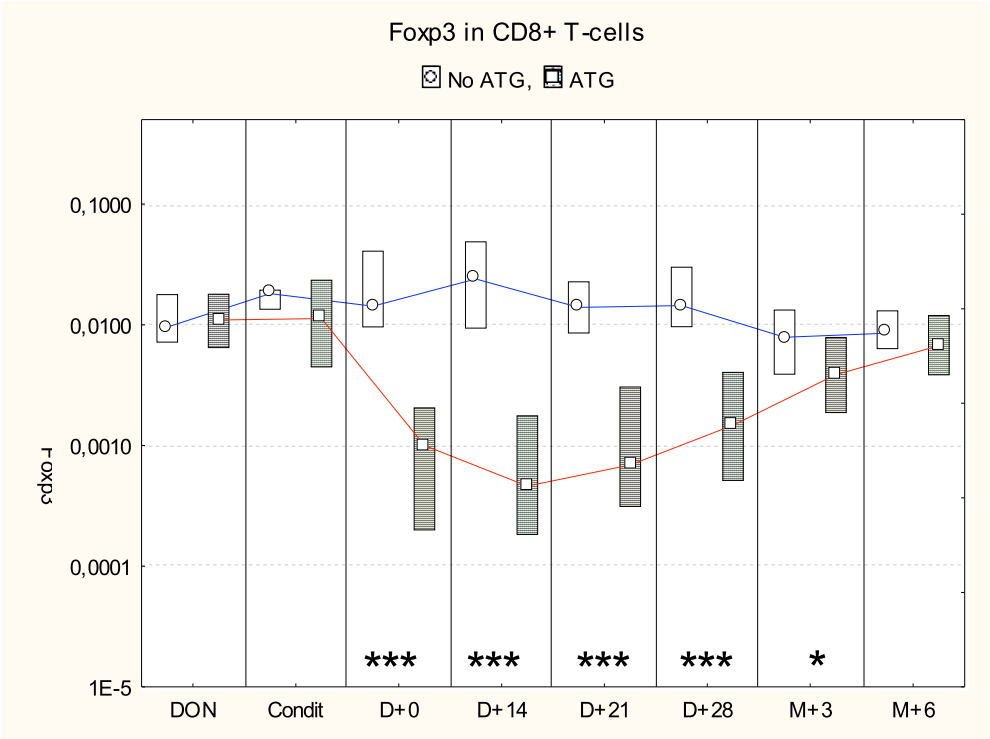


Figure 7a: Foxp3 gene expression in $CD4^+$ and $CD8^+$ T cells - comparing with (black striped) and without (white) ATG
***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$

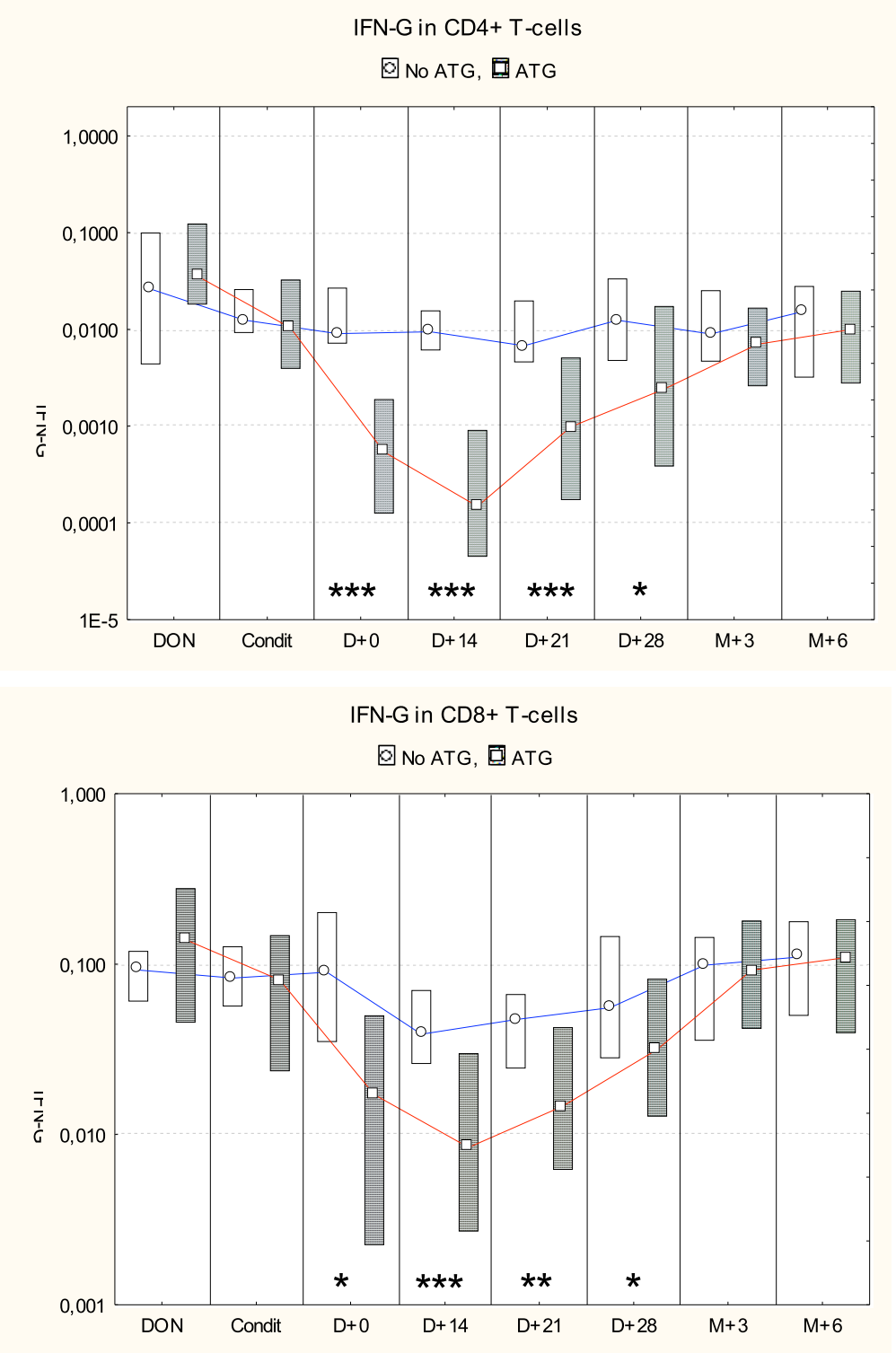


Figure 7b: IFN-γ gene expression in $CD4^+$ and $CD8^+$ T cells - comparing with (black striped) and without (white) ATG
***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$

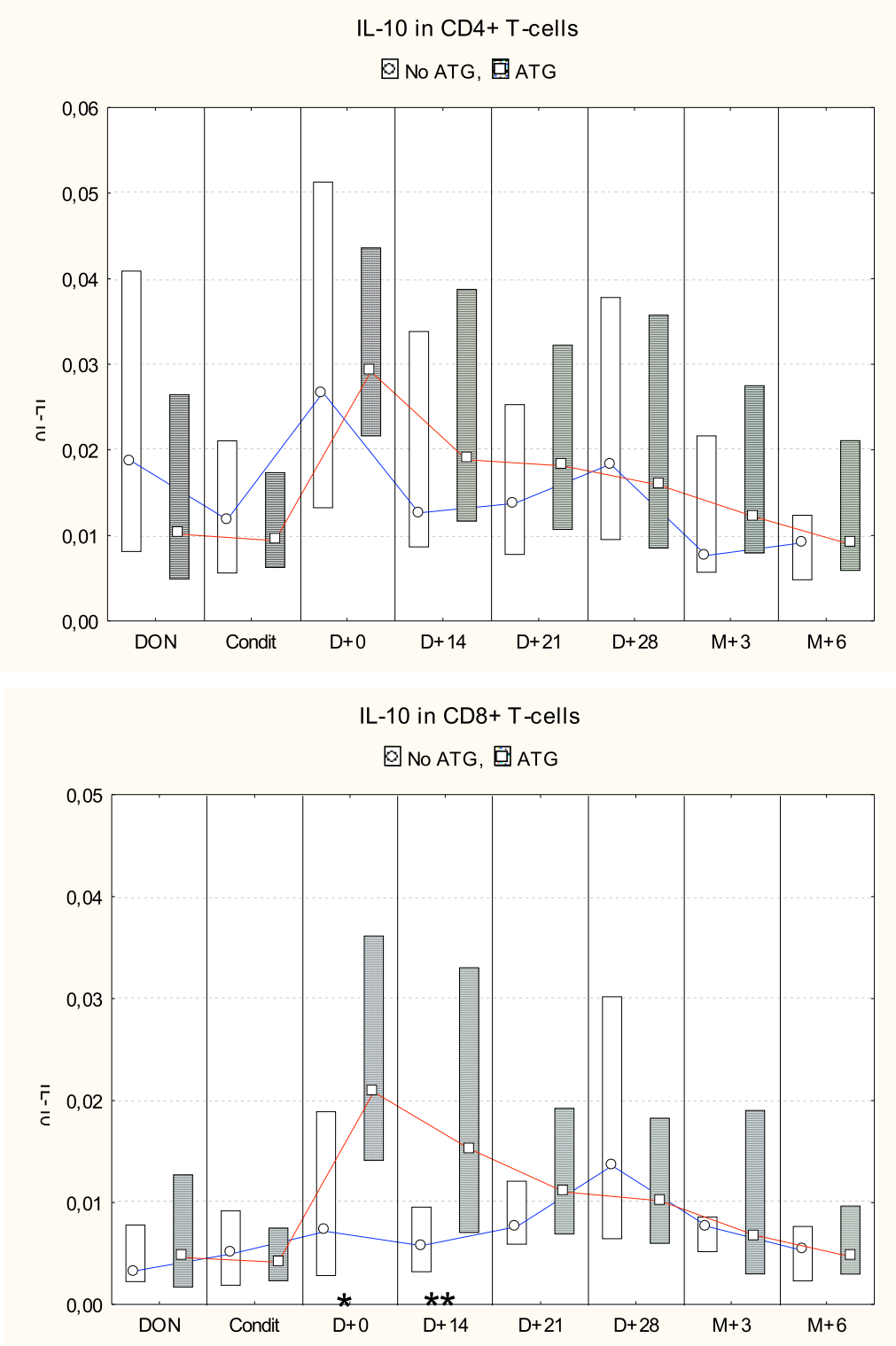


Figure 7c: IL-10 gene expression in $CD4^+$ and $CD8^+$ T cells - comparing with (black striped) and without (white) ATG
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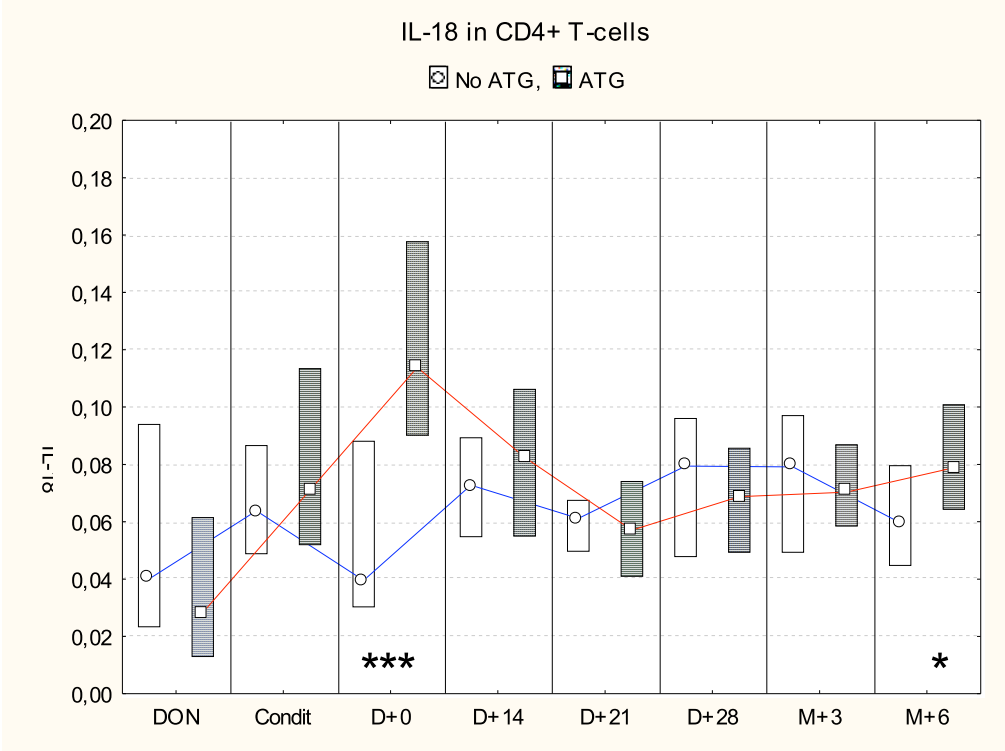

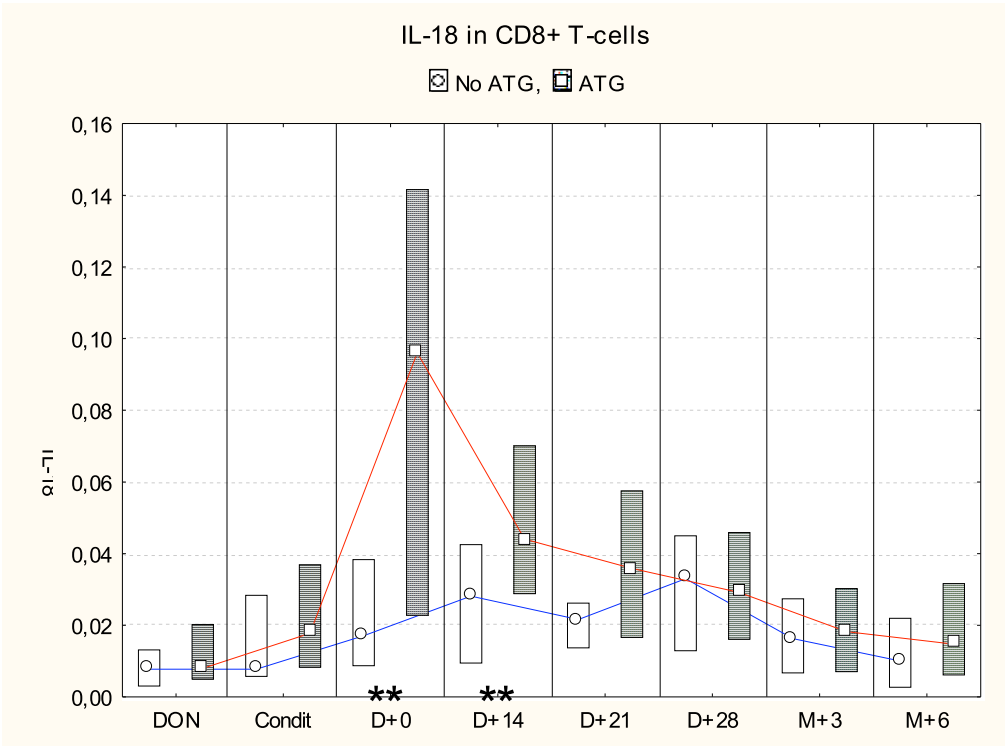

Figure 7d: IL-18 gene expression in $CD4^+$ and $CD8^+$ T cells - comparing with (black striped) and without (white) ATG
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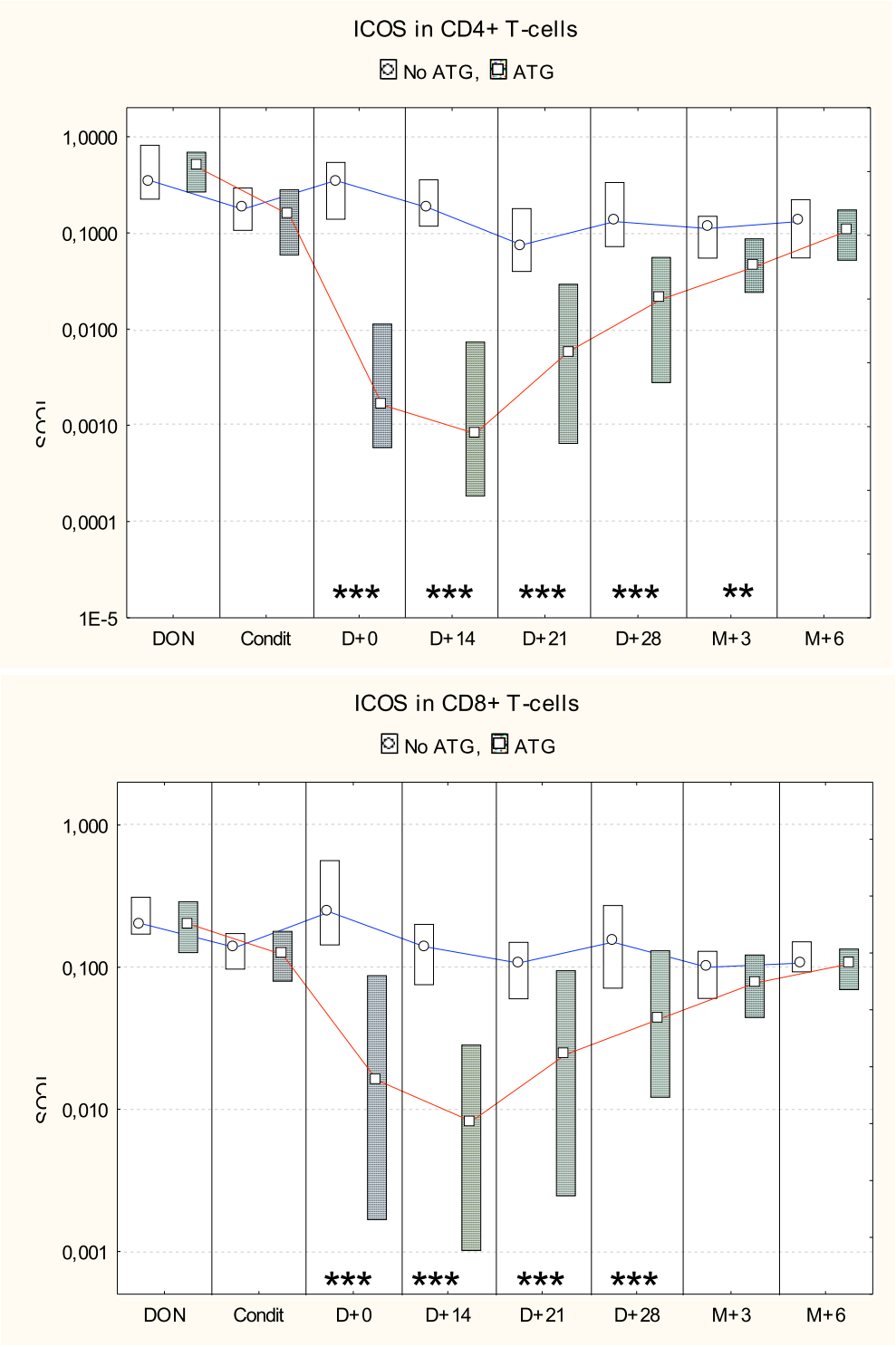
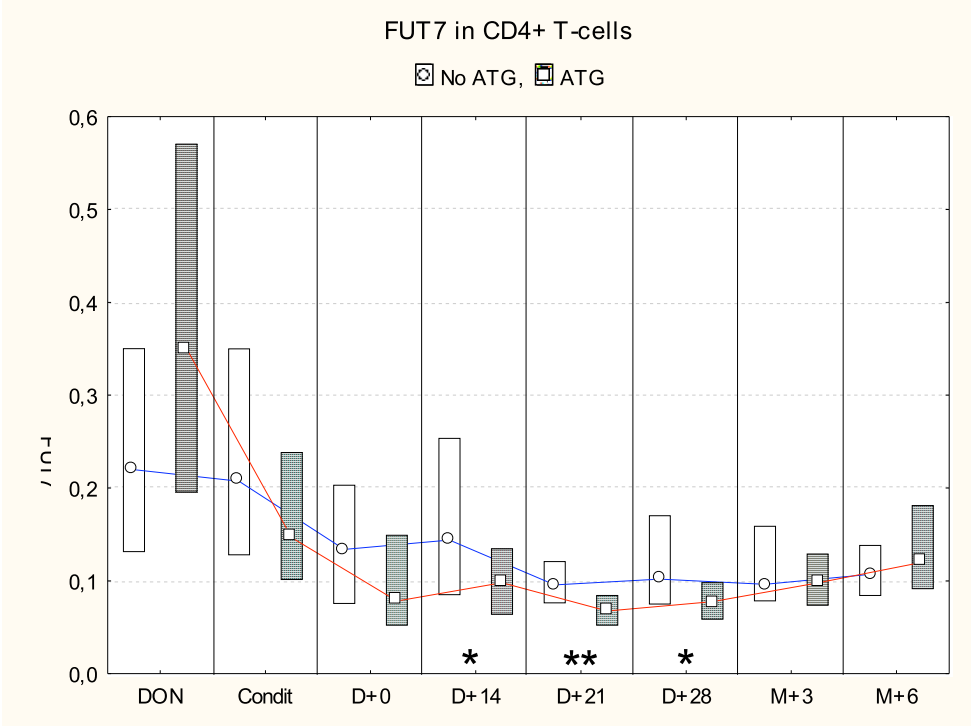


Figure 7e: ICOS gene expression in $CD4^+$ and $CD8^+$ T cells - comparing with (black striped) and without (white) ATG
***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$



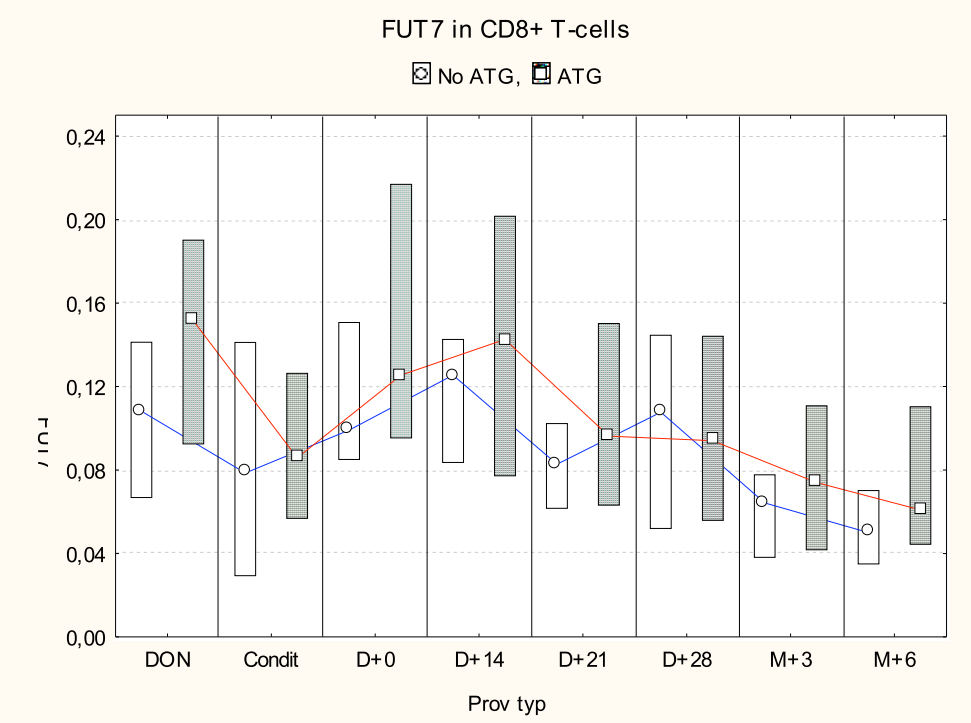


Figure 7f: FuT7 gene expression in $CD4^+$ and $CD8^+$ T cells - comparing with (black striped) and without (white) ATG
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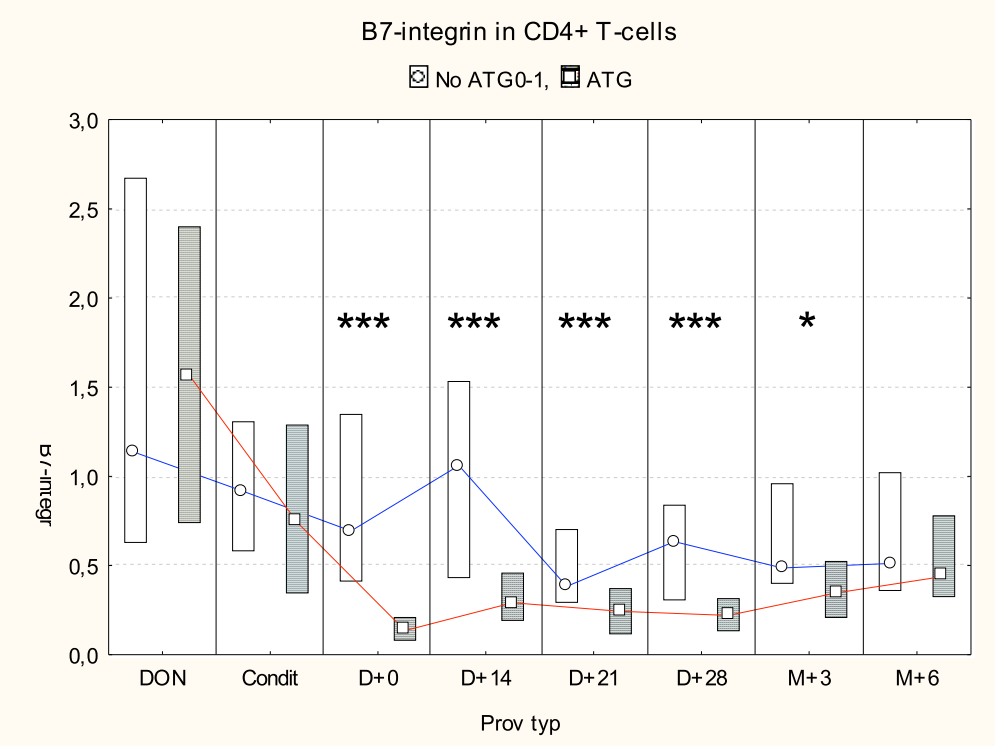


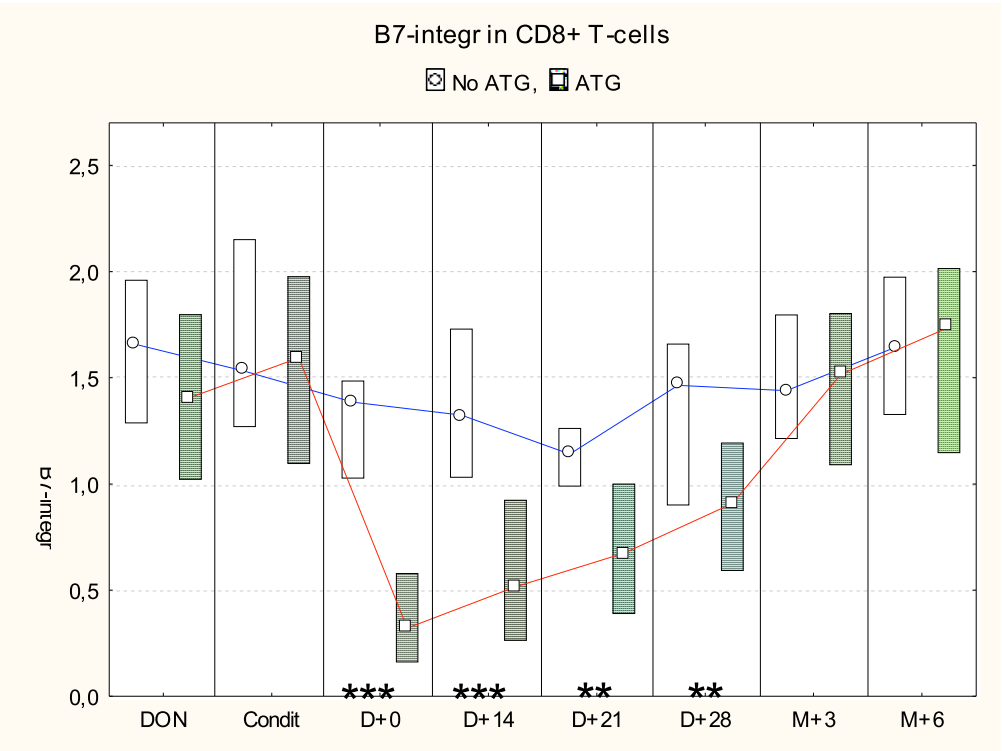


Figure 7g: B7-int gene expression in $CD4^+$ and $CD8^+$ T cells - comparing with (black striped) and without (white) ATG
***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$

The most significant differences occurred in gene expression levels of Foxp3, IFN-γ, ICOS and B7-int, in both $CD4^+$ and $CD8^+$ T cells (figure 7a-g).

Foxp3 and ICOS had a similar gene expression development from the day of transplantation to 6 months after HSCT. Without ATG the levels of Foxp3 and ICOS were quite constant in both T cell types, comparing to the donors' samples and pre-conditioning states, whereas both Foxp3 and ICOS were significantly decreased in patients with ATG treatment. In $CD4^+$ T cells the gene expression levels of Foxp3 and ICOS were lowered up to 100-times with ATG ($p < 0.001$), but the expressions started to increase 21 days after transplantation again and reached the same gene expression levels as without ATG 6 months after HSCT. In $CD8^+$ T cells the mRNA levels of Foxp3 and ICOS were decreased 10-times with ATG ($p < 0.001$) and also started to increase, like in $CD4^+$ T cells at day 21. A comparable Foxp3 gene expression level of patients with and without ATG was reached after 6 months and a comparable ICOS gene expression level after 3 months in $CD8^+$ T cells.

The gene expression levels of IFN-γ were constant over time without ATG treatment in $CD4^+$ T cells, but slightly decreased in $CD8^+$ T cells. A strong IFN-γ decrease occurred with T cell-depleted transplants, up to 100-times in $CD4^+$ T cells ($p < 0.001$) and up to 10-times in $CD8^+$ T cells ($p < 0.01 – 0.001$). The mRNA level of IFN-γ was increased at day 21 after transplantation in both T cell types and reached a normal level, corresponding to donors' value and patients' pre-conditioning state, after 3 months.

In $CD4^+$ T cells the gene expression of B7-int was slightly decreased without ATG 21 days after transplantation. 6 months after HSCT the B7-int mRNA levels of patients with non-T cell-depleted transplants was still lowered, comparing to pre-conditioning state. In $CD8^+$ T cells of patients without ATG administration the mRNA levels of B7-int was almost constant. In both $CD4^+$ and $CD8^+$ T cells the gene expressions of B7-int was significantly decreased in ATG treated patients ($p < 0.01 – 0.001$). The levels started to increase in $CD8^+$ T cells 14 days after transplantation again.

The gene expression levels of FuT7 hardly differed in both T cell types comparing patients with and without ATG. However, the mRNA level of FuT7 was decreased with ATG from day 14 to day 28 after transplantation in $CD4^+$ T cells ($p < 0.05 – 0.01$). Furthermore the overall expression was rather constant.

The mRNA level of IL-10 was quite constant in $CD8^+$ T cells without ATG, but was slightly increased at the day of transplantation and 14 days after with ATG ($p < 0.05 – 0.01$). In $CD4^+$ T cells IL-10 was mildly increased at the day of transplantation, not mattering if the transplant was T cell-depleted or not.

The gene expression level of IL-18 was slightly increased in $CD8^+$ T cells during the time period after conditioning to day 28 after transplantation without ATG, whereas IL-18 was significantly more increased at the day of transplantation and 14 days after with ATG ($p < 0.01$). In $CD4^+$ T cells the IL-18 gene expression was decreased at the day of transplantation without ATG, but significantly increased with ATG administration ($p < 0.001$). In $CD4^+$ T cells IL-18 was expressed similarly in patients with and without ATG from 14 days to 6 months after HSCT.

5.1.3 Gene Expression Levels in Donor T Cells - BMSC versus PBSC

Maybe the stem cell source was important for a diverse expression of the genes and thus for differences in aGVHD development. We compared therefore the donors' gene expression levels of BMSC with PBSC grafts, as shown in figure 8a-b. CBSC only delivered a few amount of cells, which all were used for transplantation and not for further studies. Comparing the gene expression levels of BMSC donor grafts with PBSC donor grafts delivered some significant differences.

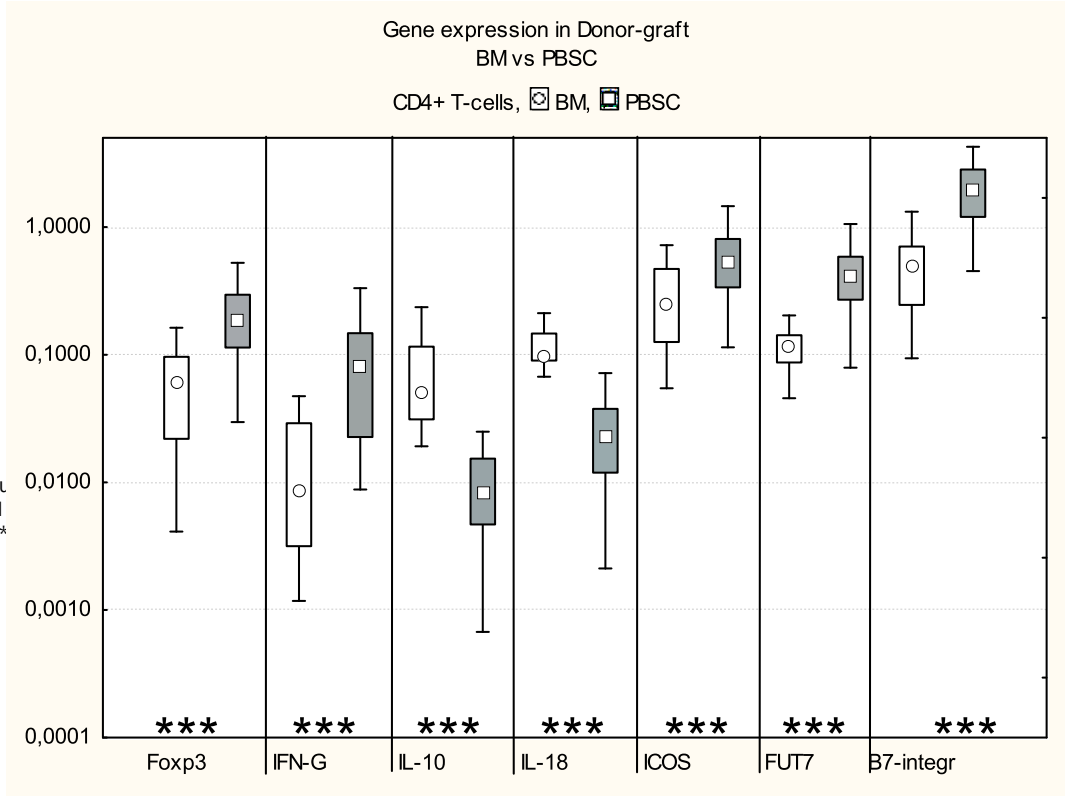


Figure 8a: Gene expression levels in donor graft $CD4^+$ T cells – comparing BMSC (white) with PBSC (black striped)
BM – bone marrow; PBSC – peripheral blood stem cells
***: $p < 0.001$; **: $p < 0.01$

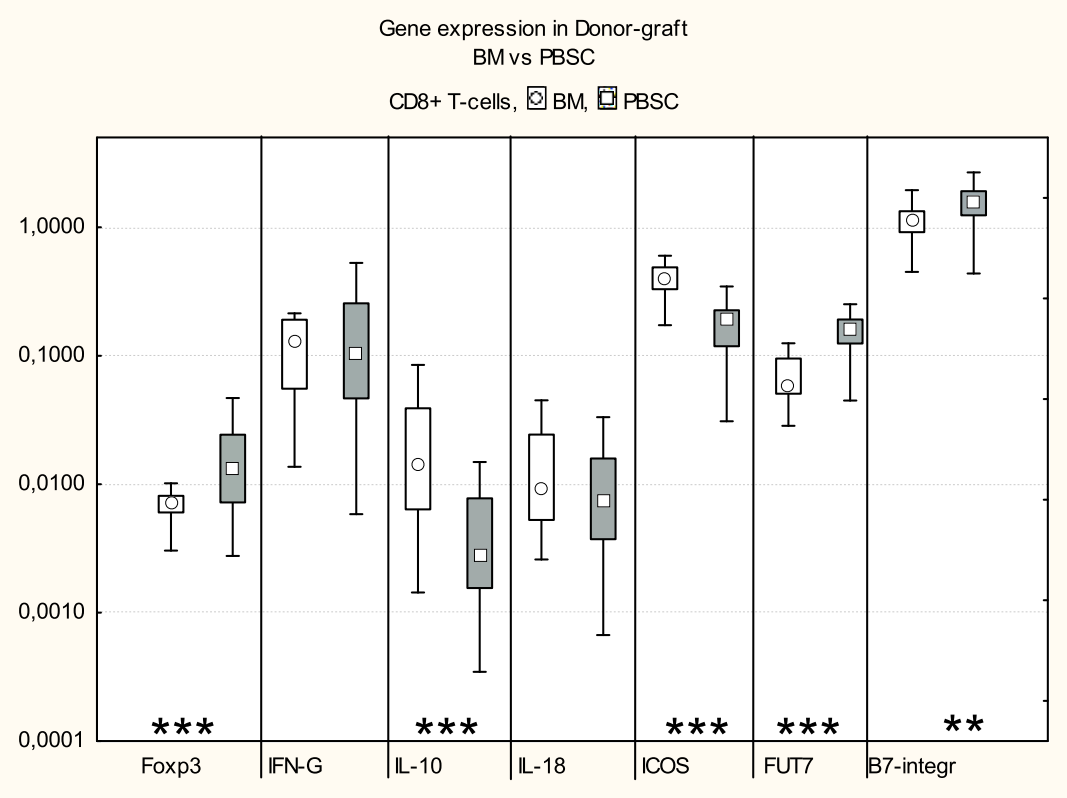


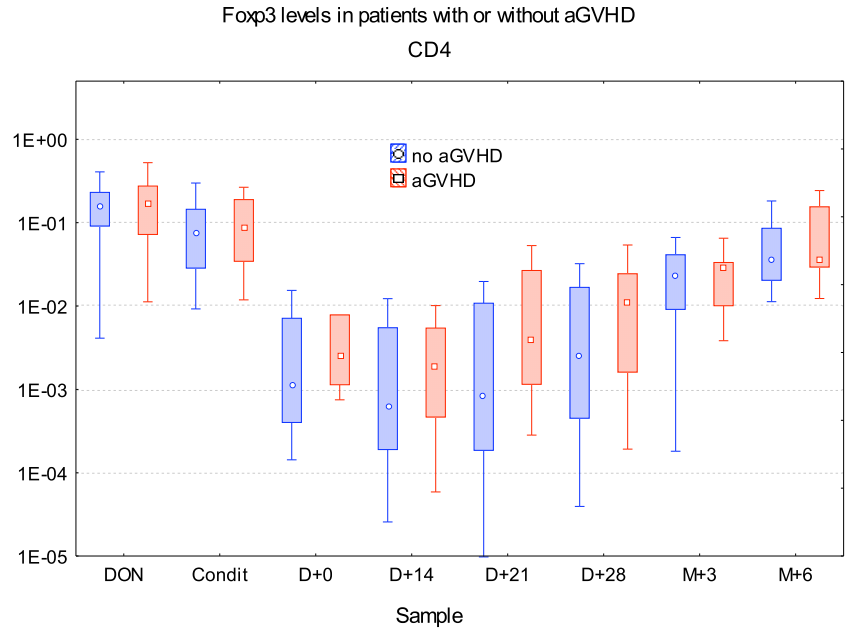
Figure 8b: Gene expression levels in donor graft CD8⁺ T cells – comparing BMSC (white) with PBSC (black striped)
BM – bone marrow; PBSC – peripheral blood stem cells
***: $p < 0.001$; **: $p < 0.01$

In CD4⁺ T cells Foxp3, IFN-γ, ICOS, FuT7 and B7-int were significantly higher expressed in donor samples of PBSC grafts than in samples of BMSC grafts ($p < 0.001$), whereas IL-10 and IL-18 were significantly lower expressed ($p < 0.001$). Foxp3, FuT7 and B7-int also had a significantly higher and IL-10 a significantly lower expression ($p < 0.001$) in CD8⁺ T cells deriving from PBSC donor grafts than those from BMSC grafts, like in CD4⁺ T cells. IFN-γ and IL-18 were in contrast equally expressed in CD8⁺ T cells of both different stem cell sources. In CD8⁺ T cells ICOS was higher expressed in blood samples of BMSC donor grafts than in samples of PBSC grafts, inversely to CD4⁺ T cells, resulting that the overall ICOS expression was equal in both different donor graft types.

These gene expression differences of the two diverse stem cell sources were only seen in the donors' but not patients' samples (data not shown). Additionally, the used stem cell source had no influence on the development of aGVHD. 45% of the patients, who got BMSC transplants and 52% receiving PBSC transplants developed aGVHD.

5.1.4 Gene Expression Levels in T Cells - with and without GVHD

We compared the gene expression levels of Foxp3, IFN-γ, IL-10, IL-18, ICOS, FuT7 and B7-int with help of RQ-PCR in patients, who did not develop aGVHD with patients, who got aGVHD, to see if there were any significant differences between those 2 groups. Th us, cytokines that act as diagnostic markers or as therapeutic approaches could maybe identified. Only patients' sampels with ATG treatment were analyzed, because ATG, as seen before, had a huge influence on the gene expressions, so that altered expression levels were due to aGVHD and not to ATG.



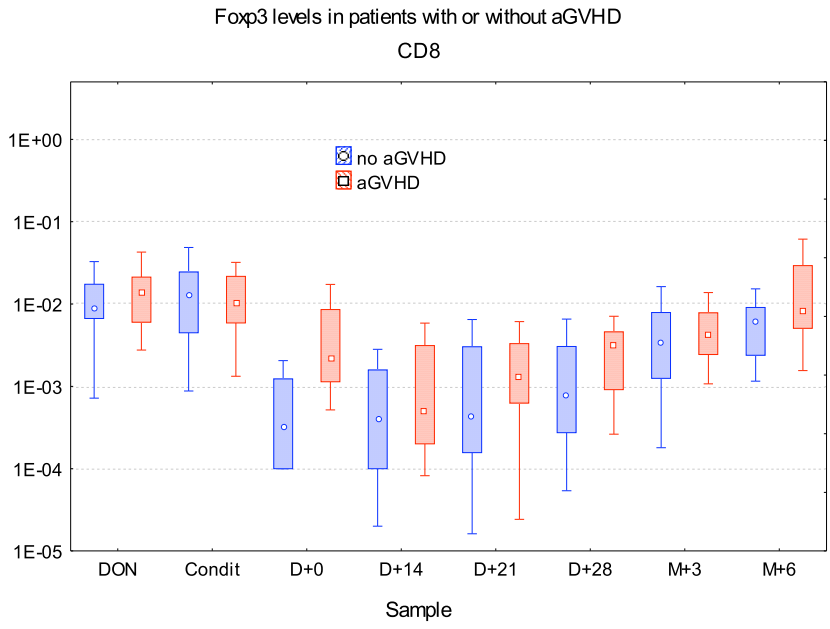
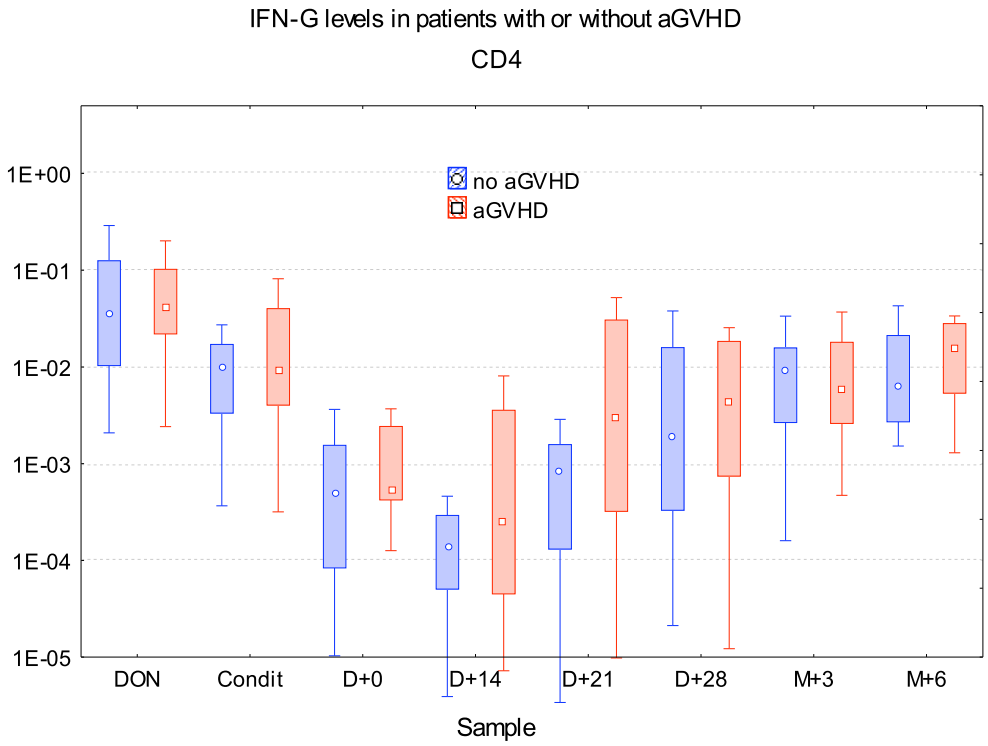


Figure 9a: Foxp3 gene expression levels in $CD4^+$ and $CD8^+$ T cells – comparing patients with (red) and without (blue) aGVHD
DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT



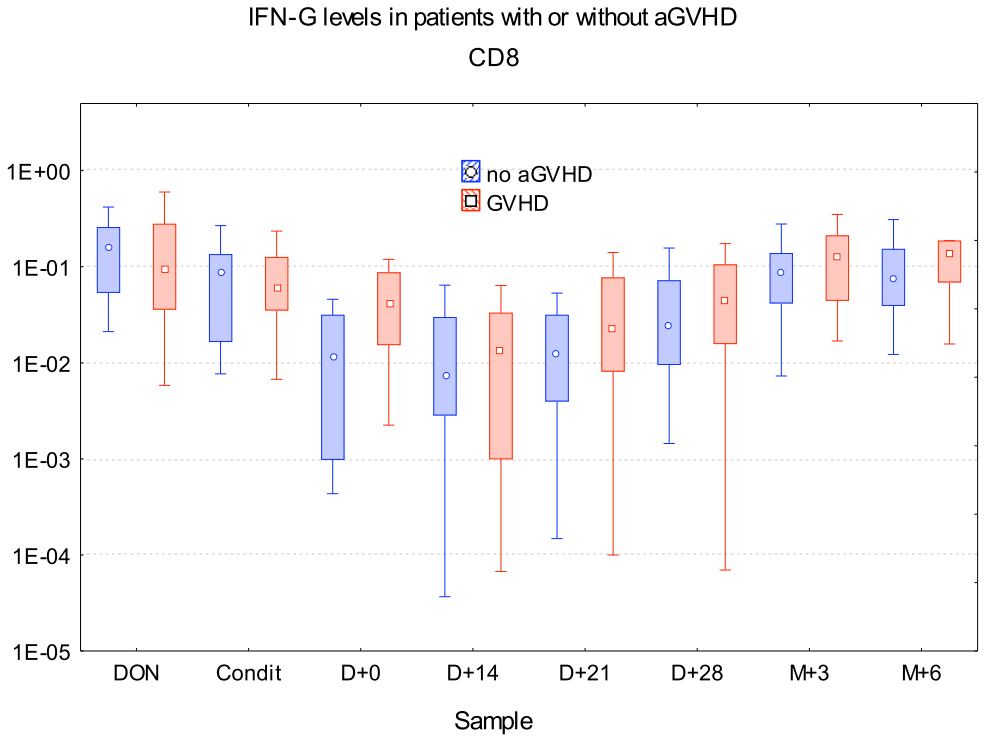


Figure 9b: IFN-γ gene expression levels in $CD4^+$ and $CD8^+$ T cells – comparing patients with (red) and without (blue) aGVHD
DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

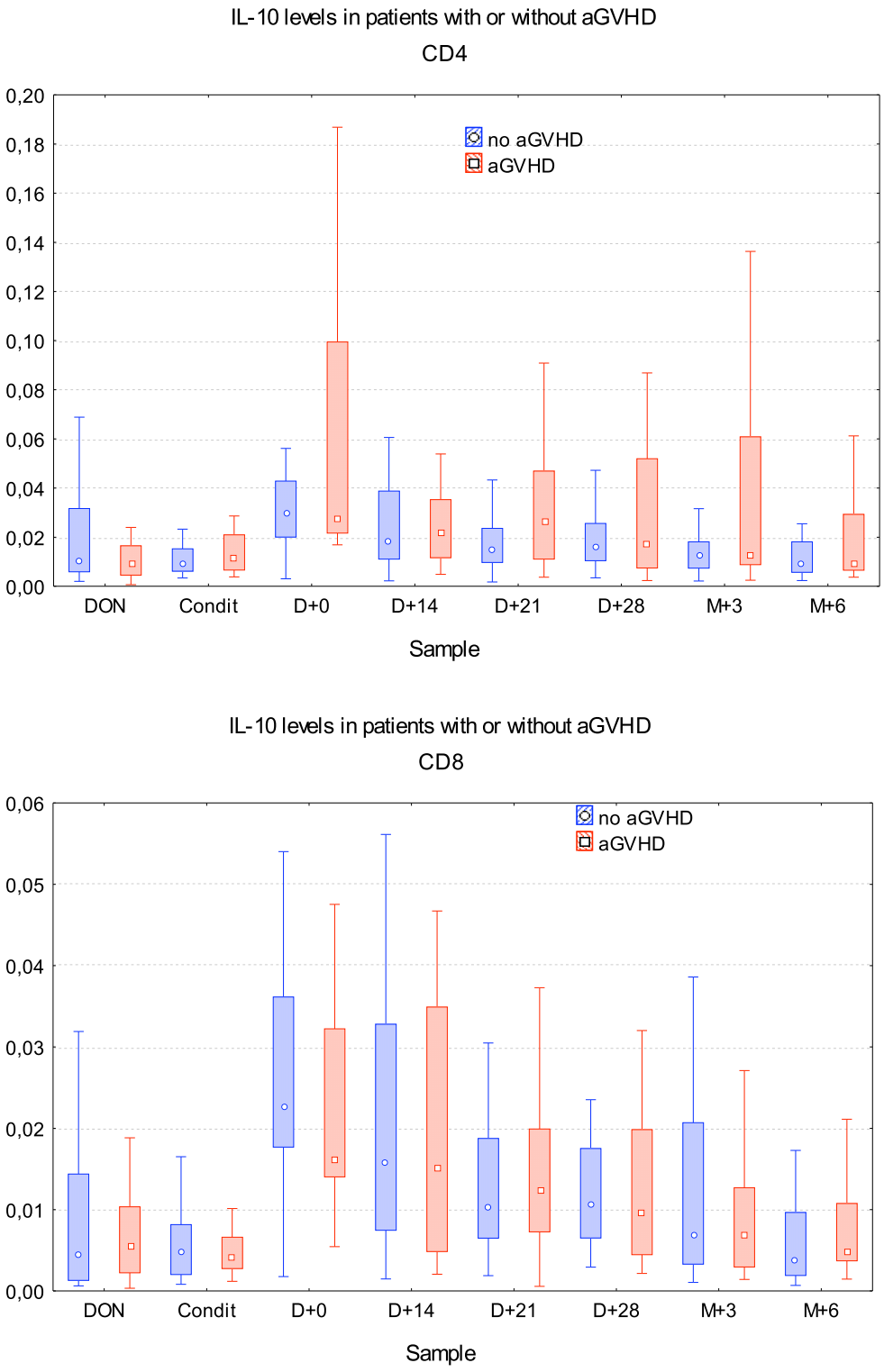


Figure 9c: IL-10 gene expression levels in $CD4^+$ and $CD8^+$ T cells – comparing patients with (red) and without (blue) aGVHD
DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

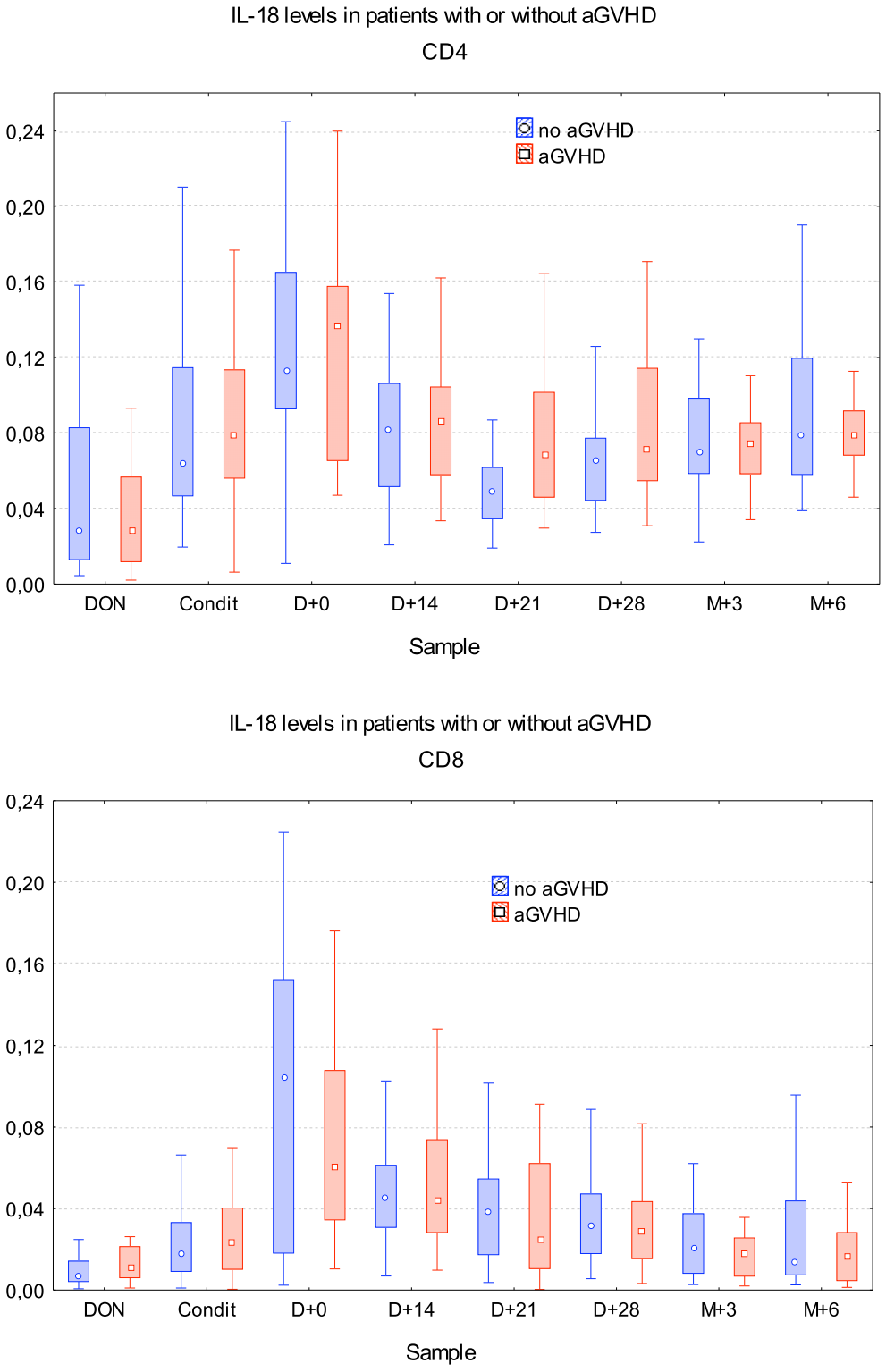


Figure 9d: IL-18 gene expression levels in $CD4^+$ and $CD8^+$ T cells – comparing patients with (red) and without (blue) aGVHD
DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

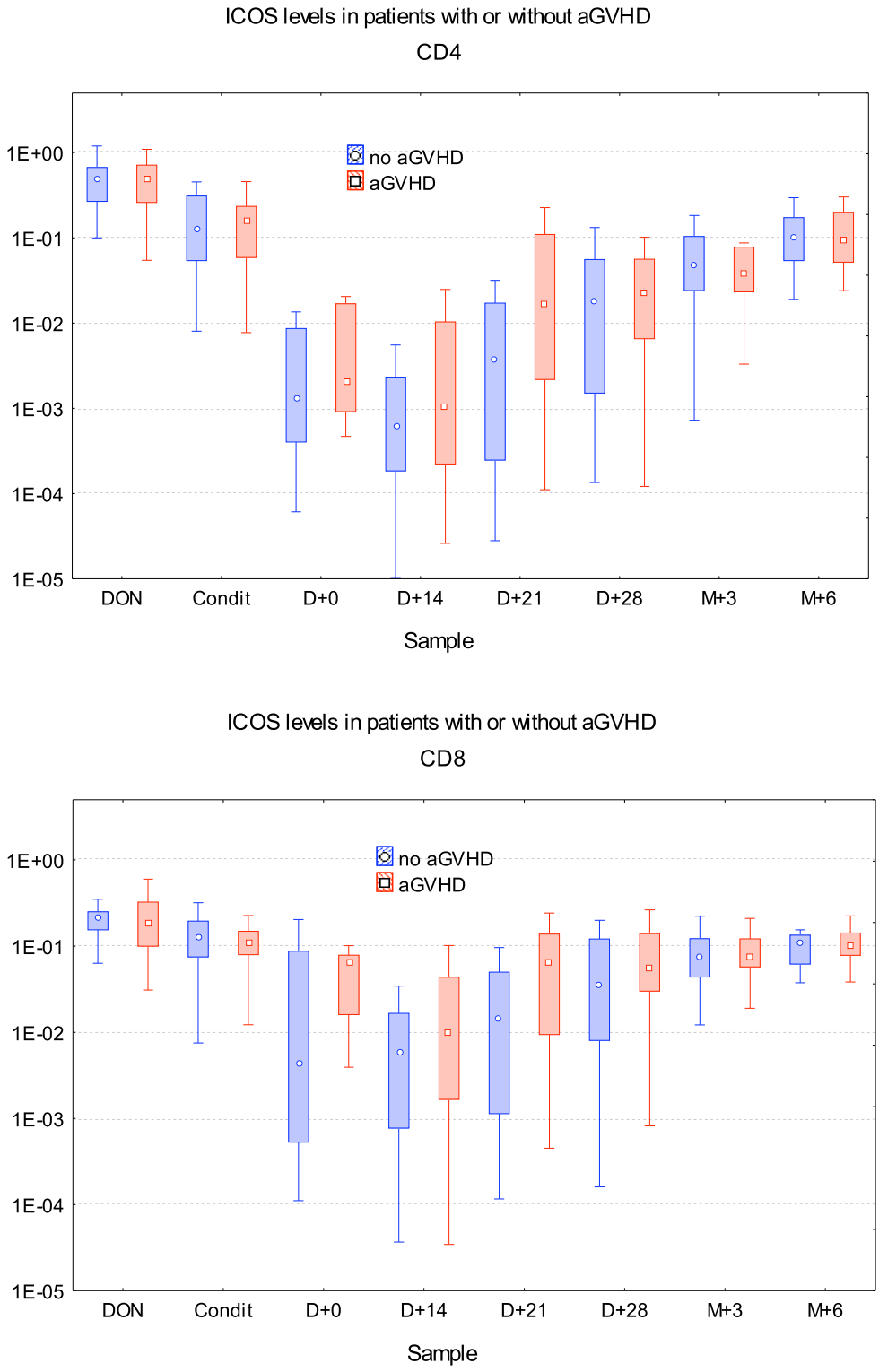
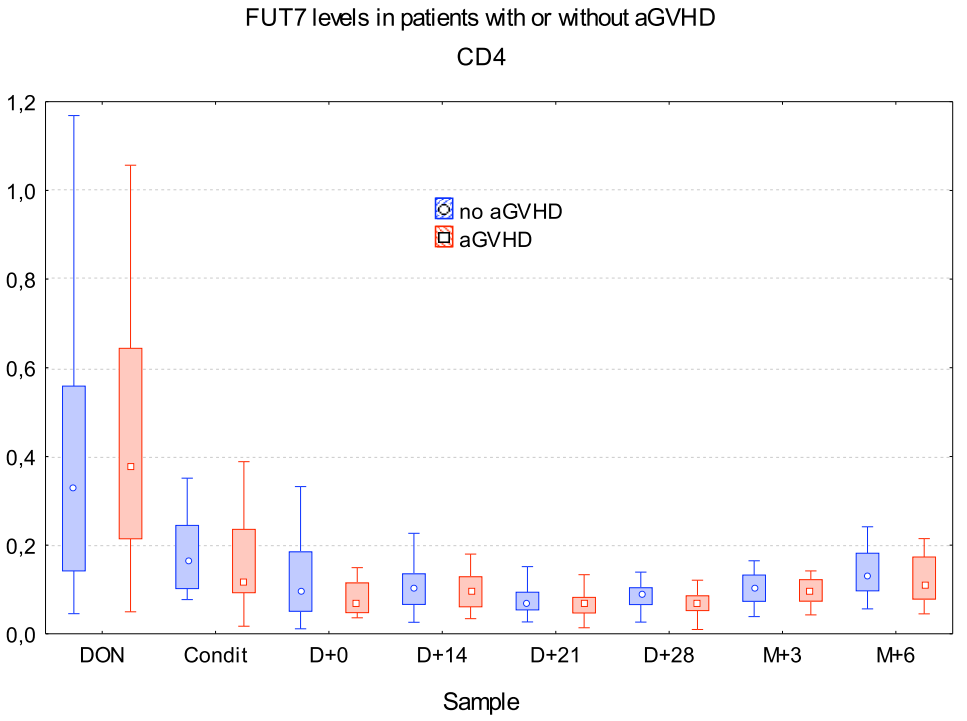


Figure 9e: ICOS gene expression levels in $CD4^+$ and $CD8^+$ T cells – comparing patients with (red) and without (blue) aGVHD
DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT



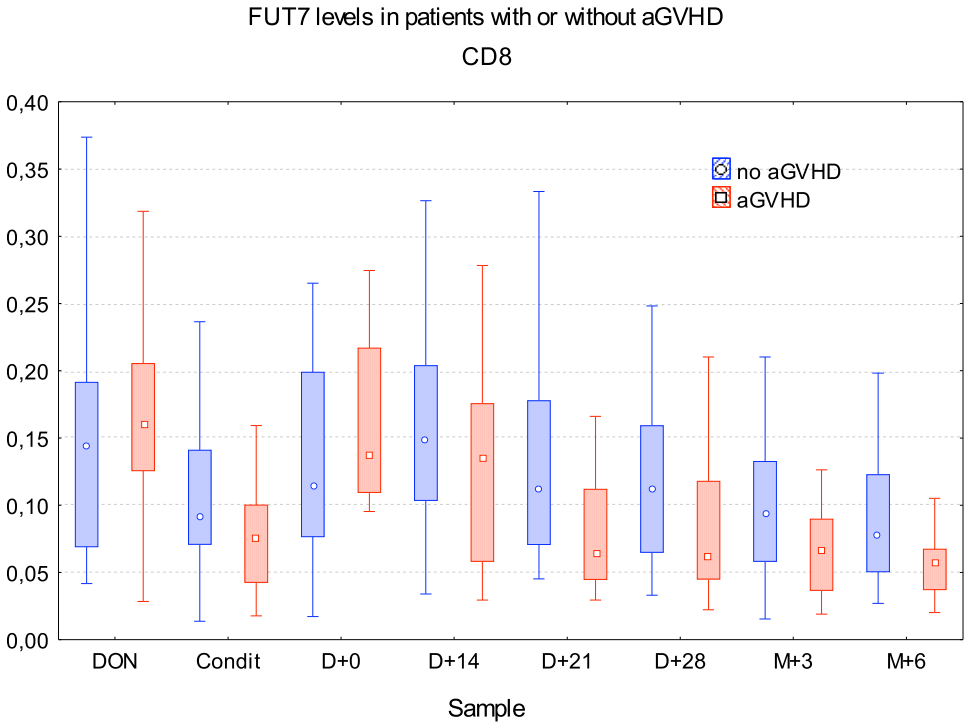


Figure 9f: FuT7 gene expression levels in $CD4^+$ and $CD8^+$ T cells – comparing patients with (red) and without (blue) aGVHD
DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

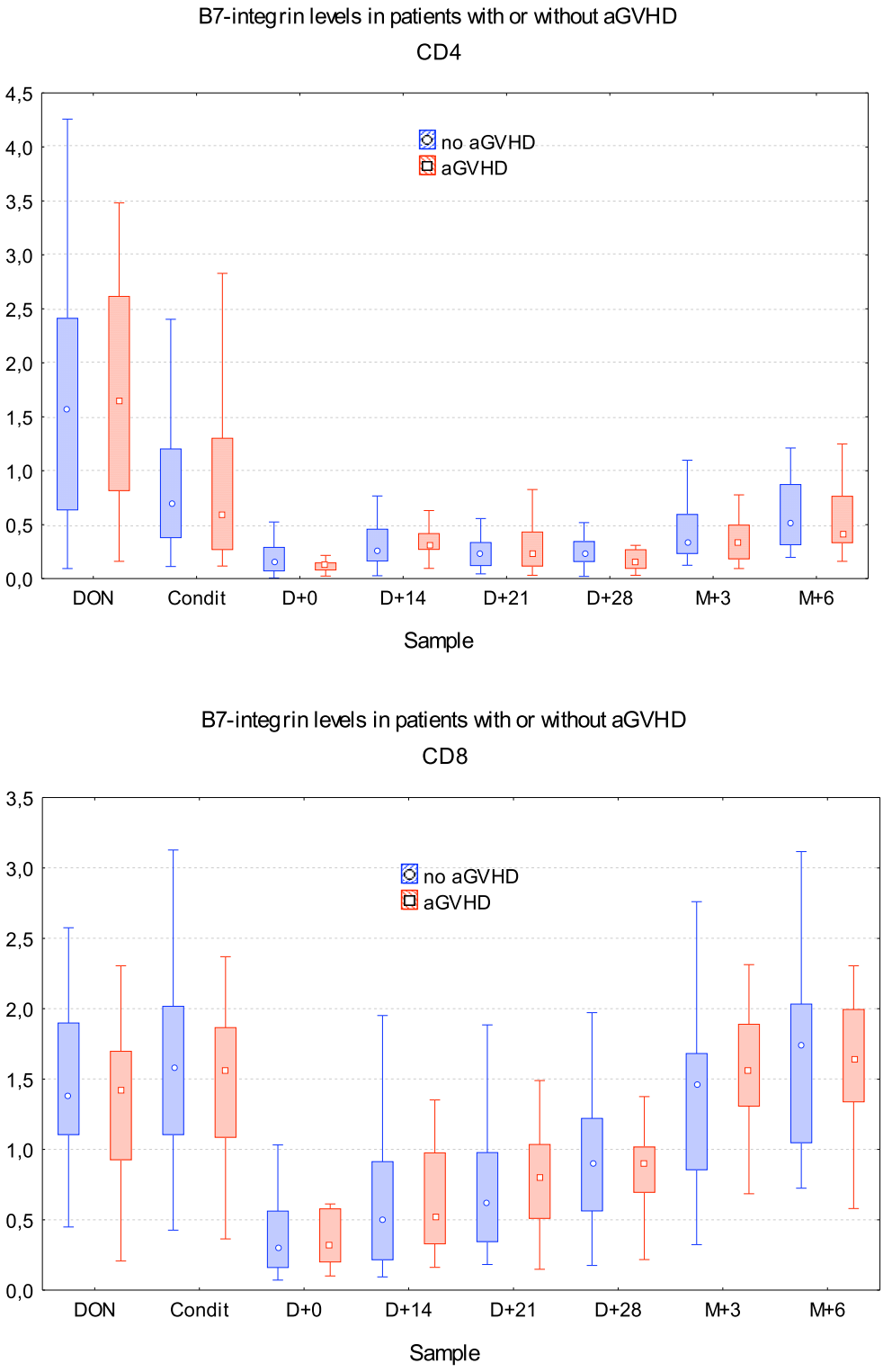


Figure 9g: B7-int gene expression levels in $CD4^+$ and $CD8^+$ T cells – comparing patients with (red) and without (blue) aGVHD
DON, donor; Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

The gene expression levels in the 2 different patient groups did not show any significant differences regarding IL-10 or B7-int in $CD4^+$ and $CD8^+$ T cells (figure 9c+g), respectively. However, we measured some significant expression differences in the other analyzed genes. Foxp3 (figure 9a) was significantly more expressed in $CD8^+$ T cells of patients with aGVHD comparing to patients without aGVHD at the day of transplantation ($p = 0.005$) and 28 days after HSCT ($p = 0.020$). Furthermore was a trend seen towards more Foxp3 expression in $CD8^+$ T cells of patients with aGVHD 6 months after transplantation ($p = 0.053$). In $CD4^+$ T cells Foxp3 was significantly more expressed in patients with aGVHD 21 days after transplantation ($p = 0.016$), although no trend was detected at any other timepoint. IFN-γ (figure 9b) had significantly higher gene expression levels in $CD4^+$ T cells of patients without aGVHD 21 days after HSCT ($p = 0.033$). However no such significance was detected in $CD8^+$ T cells, nor was a trend seen at any other timepoint occasion. Patients, who developed aGVHD had increased IL-18 gene expression (figure 9d) in $CD4^+$ T cells 21 days after HSCT compared to patients without aGVHD ($p = 0.008$), although no trend could be measured at any other timepoint. There were no significantly different IL-18 gene expressions in $CD8^+$ T cells between the 2 patient groups. ICOS (figure 9e) was elevated in both $CD4^+$ and $CD8^+$ T cells of patients with aGVHD development 21 days after transplantation ($p = 0.029$ in $CD4^+$; and $p = 0.039$ in $CD8^+$ T cells). In $CD8^+$ T cells of patients with aGVHD the gene expression of FuT7 (figure 9f) was significantly higher than in patients without aGVHD 21 and 28 days after transplantation ($p = 0.001$ at day 21; and $p = 0.023$ at day 28), whereas the expression did not alter between the 2 patient groups in $CD4^+$ T cells.

5.2 ELISA

5.2.1 Cytokine Plasma Levels - with and without GVHD

The T cells that were analyzed via RQ-PCR were not the only source for cytokine productions. Th us, diverse cytokine levels in the plasma were analyzed further with ELISA comparing ATG-treated patients with and without aGVHD to find markers for aGVHD onset as well as possible therapeutic interventions.

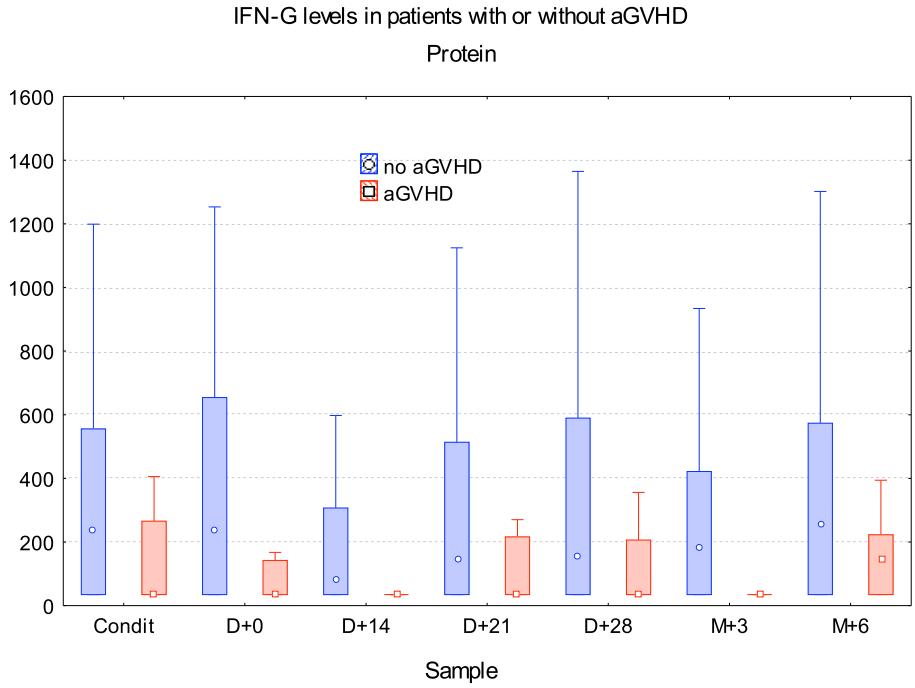


Figure 10a: IFN-γ protein plasma levels – comparing patients with (red) and without (blue) aGVHD Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

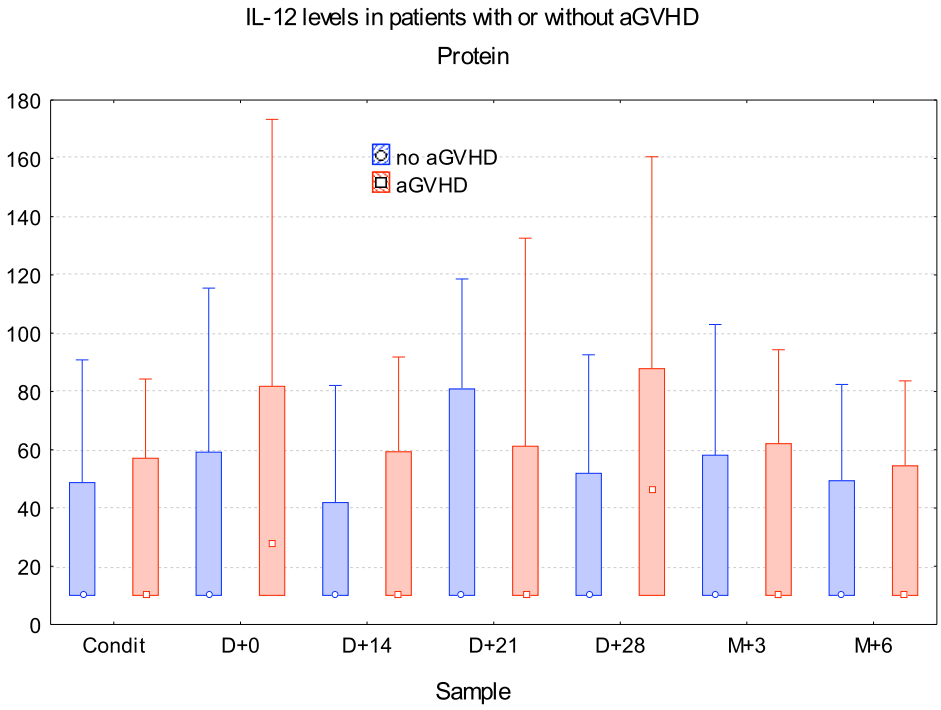


Figure 10b: IL-12 protein plasma levels – comparing patients with (red) and without (blue) aGVHD Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

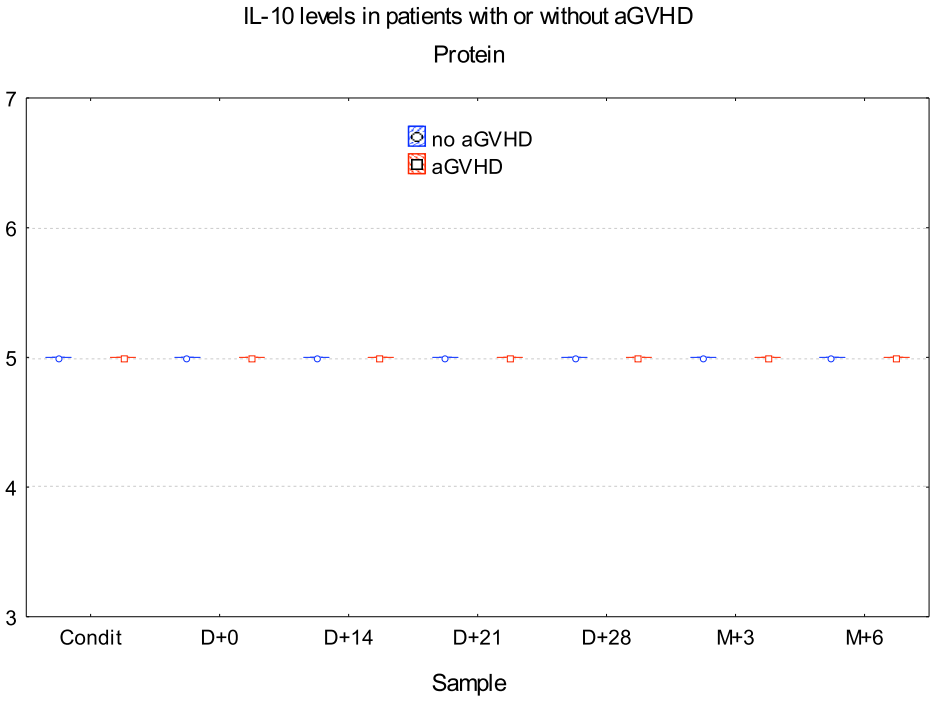


Figure 10c: IL-10 protein plasma levels – comparing patients with (red) and without (blue) aGVHD Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

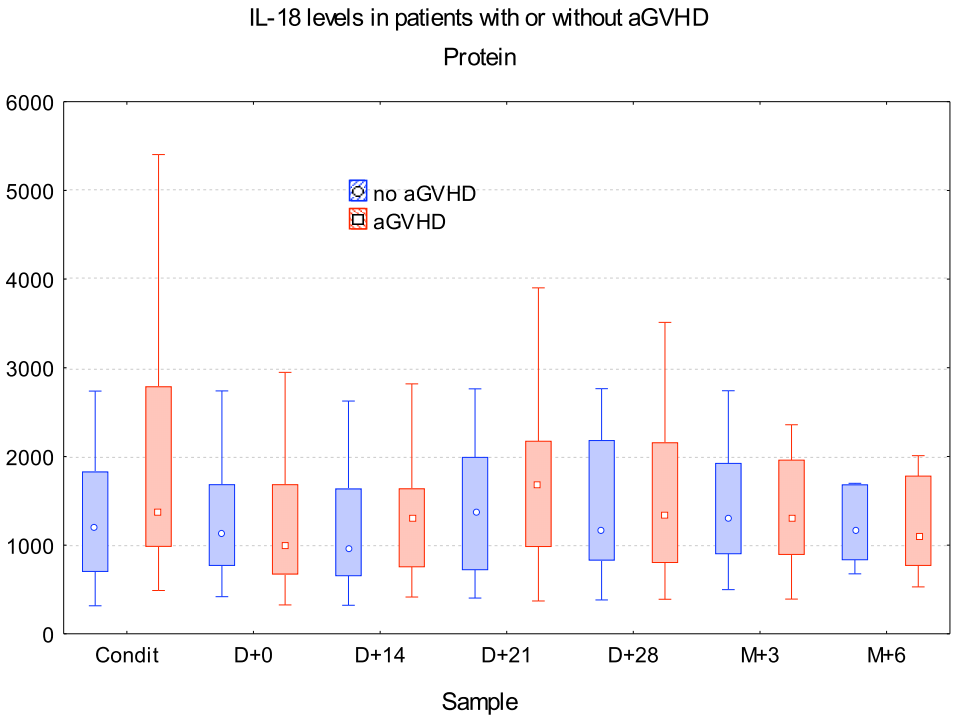


Figure 10d: IL-18 protein plasma levels – comparing patients with (red) and without (blue) aGVHD
Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

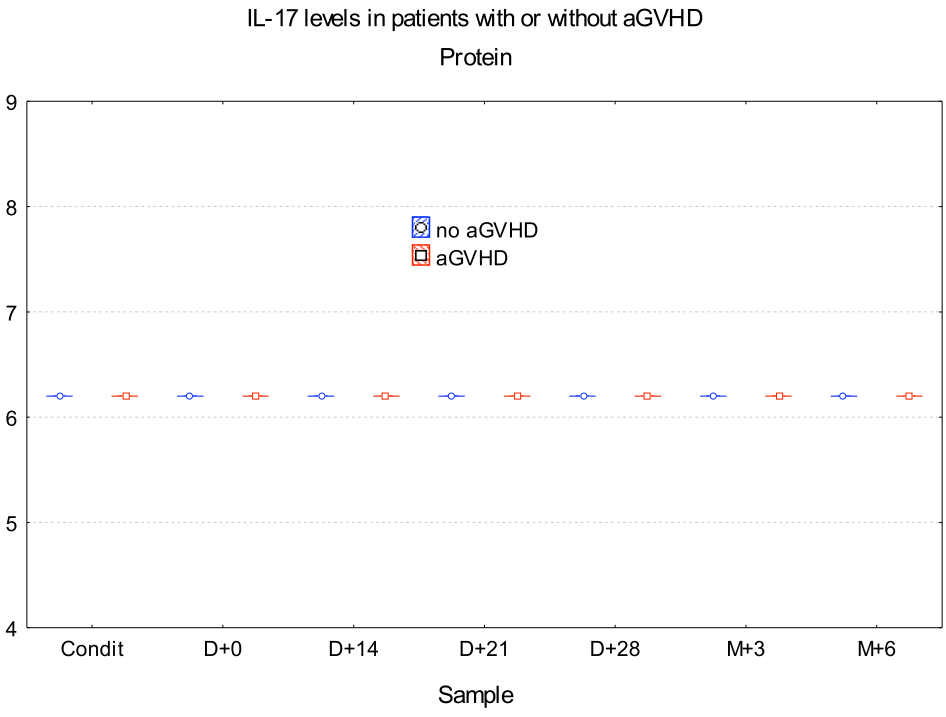


Figure 10e: IL-17 protein plasma levels – comparing patients with (red) and without (blue) aGVHD
Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

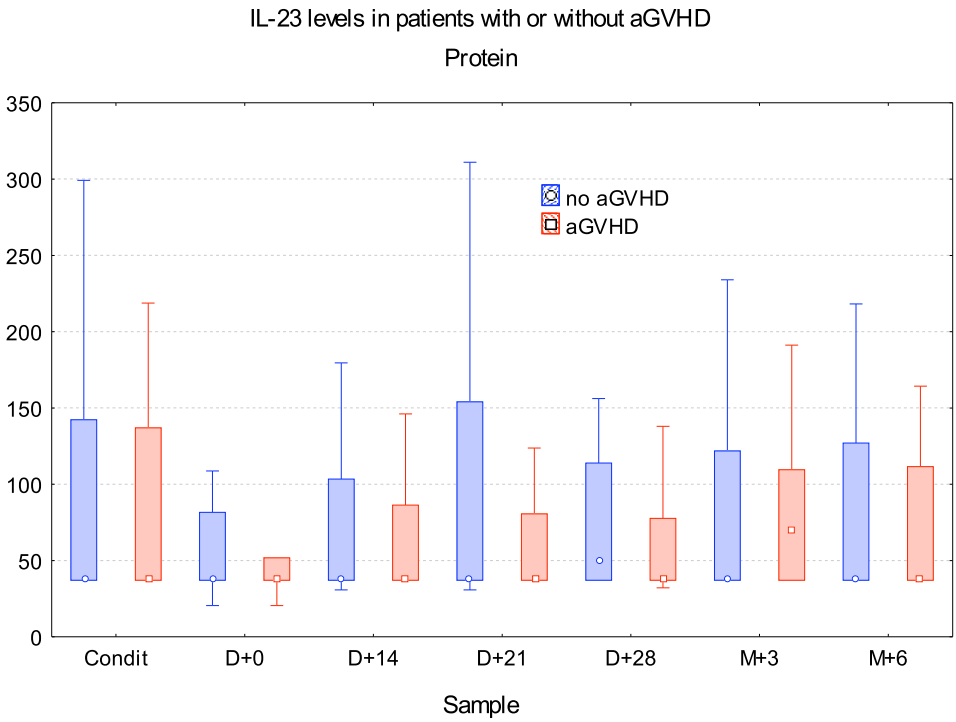


Figure 10f: IL-23 protein plasma levels – comparing patients with (red) and without (blue) aGVHD
Condt, pre-conditioning; D+0, day of HSCT; D+14/21/28, 14/21/28 days after HSCT; M+3/6, 3/6 months after HSCT

IL-10, IL-12, IL-17 and IL-23 did not deliver any significant differences in the plasma between the 2 patient groups (figure 10b,c,e,f). However, the IFN-γ plasma level (figure 10a) was significantly higher in patients without aGVHD than in patients, who developed the disease at the day of transplantation as well as 3 months after SCT (p=0.009 at day 0; p=0.037 at 3 months). There was a trend towards higher IFN-γ plasma levels in patients without aGVHD at pre-conditioning state, 14 days and 28 days after transplantation (p=0.08). Patients with aGVHD (grade I-IV) had lower IFN-γ levels at the day of transplantation (median: 35pg/ml) compared to those without aGVHD (median: 235pg/ml), shown in figure 11.

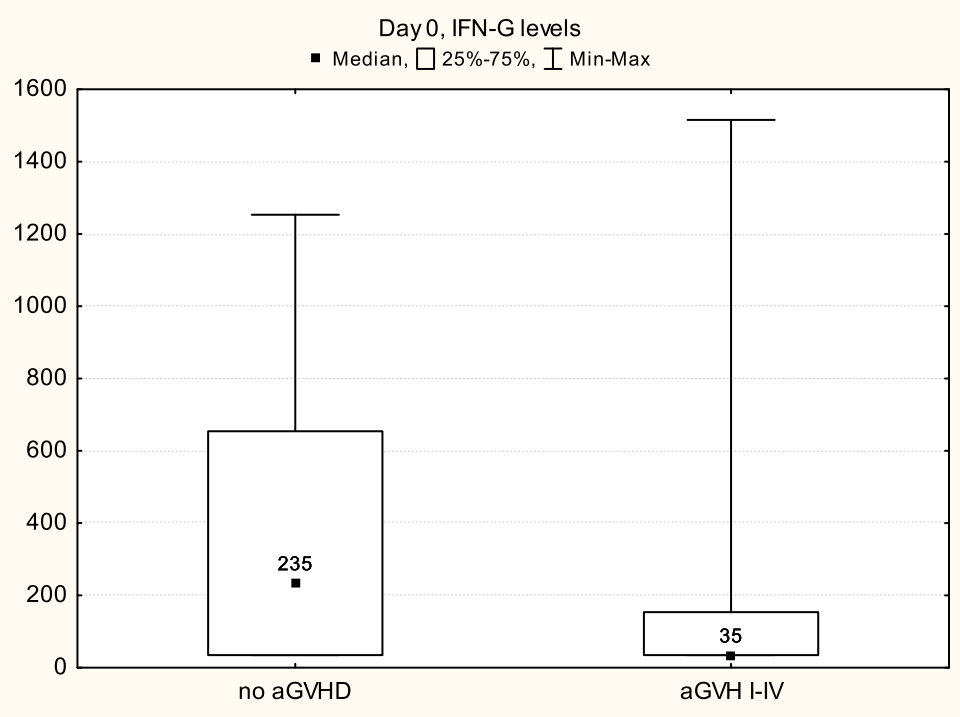


Figure 11: IFN-γ protein plasma levels at day 0, comparing patients without aGVHD (on the left) and patients with aGVHD at grad I, II, III or IV (on the right)

The IFN-γ plasma level differed also significantly between the group including patients without aGVHD + aGVHD grade I (median: 144pg/ml) and the group including patients with more severe aGVHD (grade II-IV) (median: 35pg/ml), shown in figure 12.

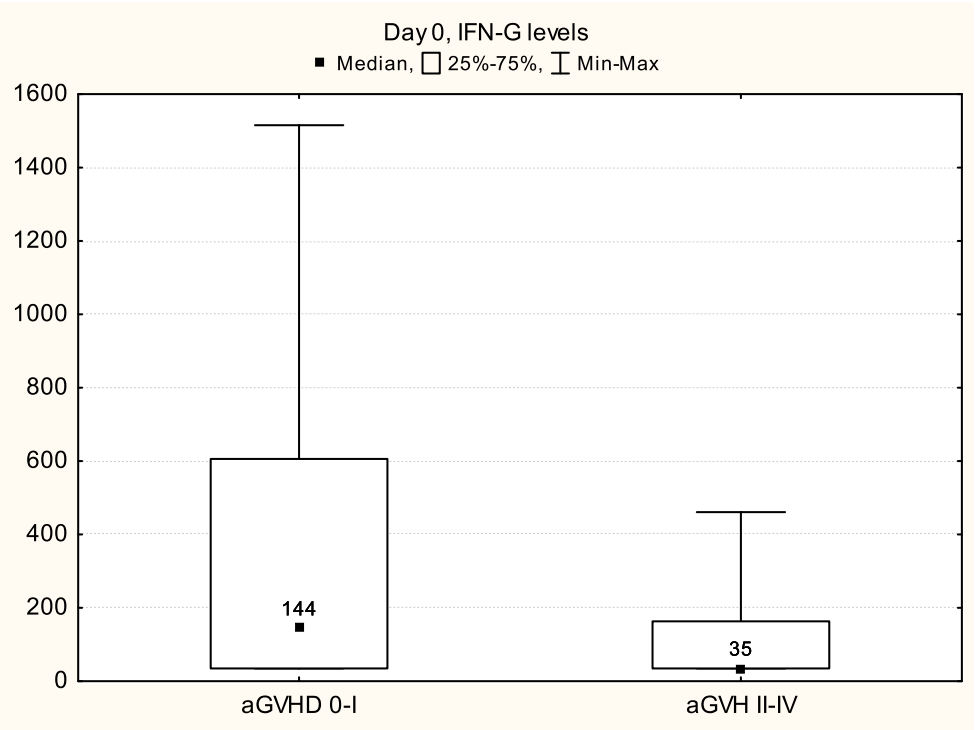


Figure 12: IFN-γ protein plasma levels at day 0, comparing patients without aGVHD or a aGVHD grad I (on the left) and patients with aGVHD grad II, III or IV (on the right)

Figure 13 shows that the incidence of getting aGVHD was much higher, if the IFN-γ plasma level was lower than 200pg/ml at the day of transplantation. 64% of the patients with an IFN-γ level lower than this threshold developed aGVHD, whereas only 17% of the patients with IFN-γ levels above this value developed the disease.

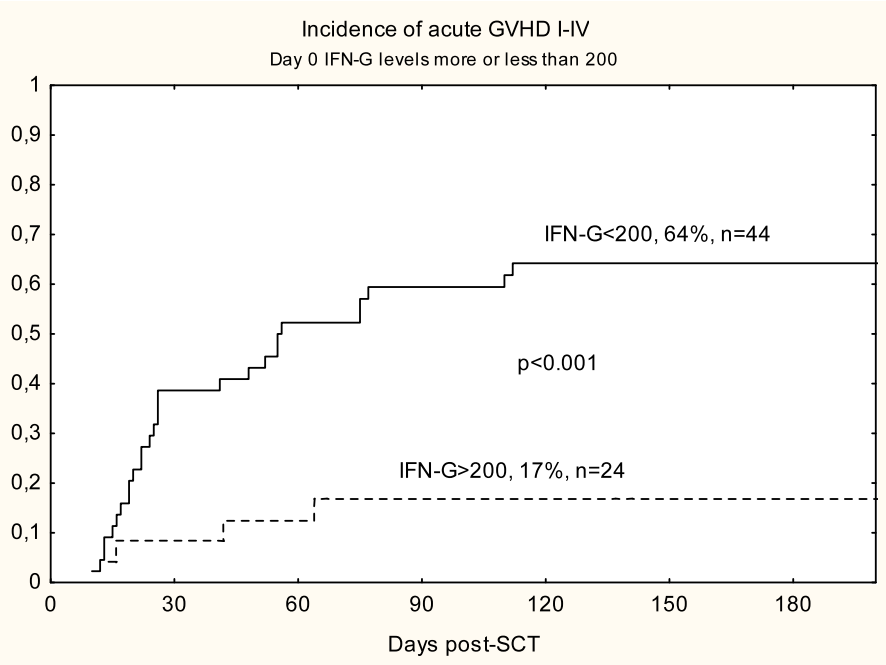


Figure 13: Incidence of aGVHD grad I-IV
comparing IFN-γ protein levels at day 0 higher and lower than 200pg/ml. 64% of the patients with low IFN-γ levels got aGVHD, whereas 17% of the patients with IFN-γ levels over 200pg/ml got aGVHD.

In figure 14 the incidence of getting aGVHD (grade II-IV) is shown. 42% of the patients with IFN-γ plasma levels lower than 200pg/ml on the day of transplantation developed aGVHD (grade II-IV), compared to 4% that had higher IFN-γ levels (>200pg/ml) in the circulation.

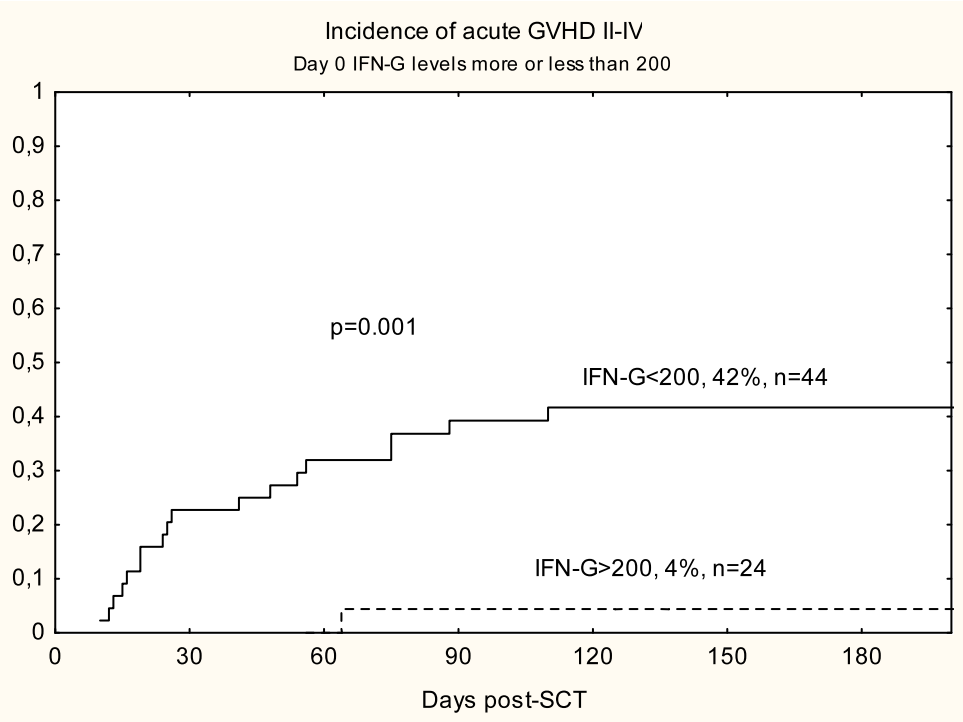


Figure 14: Incidence of aGVHD grad II-IV
comparing IFN-γ protein levels at day 0 higher and lower as 200pg/ml. 42% of the patients with low IFN-γ levels got aGVHD, whereas 4% of the patients with IFN-γ levels over 200pg/ml got aGVHD.

Comparing plasma IL-18 levels between patients with and without aGVHD at the different timepoint occasions did not deliver any significant differences. But an interesting correlation between the IL-18 plasma level and the onset day of aGVHD could be found. The IL-18 plasma levels were significantly increased in patients with aGVHD on the onset day of the disease ($p < 0.001$).

In figure 15 the IL-18 levels of all patients (with and without aGVHD) are shown. Day "0" was defined as the day of aGVHD onset. For patients, who did not develop the disease, day "0" was set as day 30 after transplantation as this was the median time for aGVHD development. The measured data of the different timepoints were related to this day "0". IL-18 was significantly elevated at the onset day of aGVHD, comparing to IL-18 levels before aGVHD development in aGVHD patients, after aGVHD onset and to patients without aGVHD.

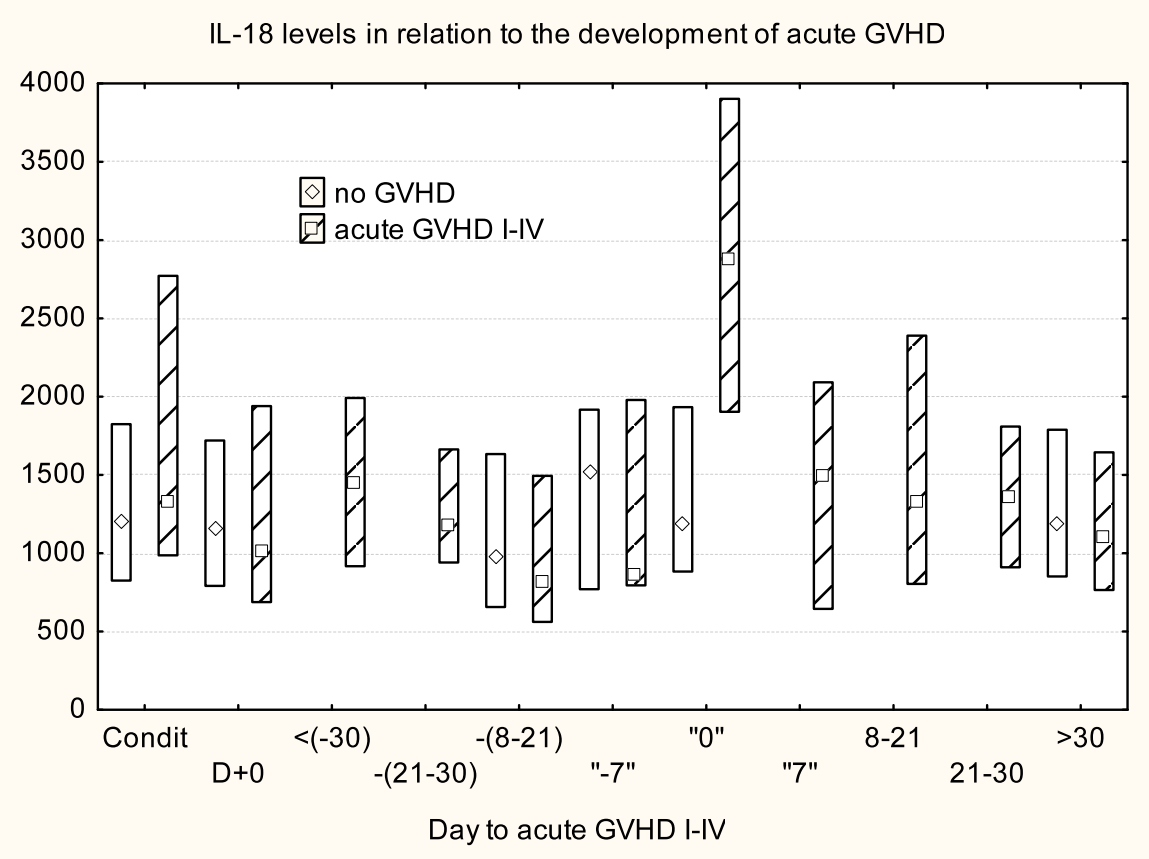


Figure 15: IL-18 protein levels in relation to the development of aGVHD
The level of IL-18 is elevated at the day of aGVHD onset, marked as day "0"

6 Discussion

6.1 Preface

To can better understand the development of GVHD and thus be able to prevent it as well as to design potential therapy forms and betimes recognition mechanisms, it is most important to know which role different cytokines play in GVHD. It is not clear yet if T_H1 cytokines enhance the development of GVHD or if they have positive effects by inhibiting the disease, or if T_H2 cytokines are the key regulators to prevent or in contrast impair GVHD, because studies with conflicting outcomes have been reported (Blazar et al., 1995; Krenger et al., 1995; Murphy et al., 1998; Yang et al. 2005).

6.2 Overall Gene Expression Levels in $CD4^+$ and $CD8^+$ T cells

In our study the equal gene expression levels of donors' and patients' pre-conditioning samples, $CD4^+$ and $CD8^+$ T cells, respectively, indicated that an altered gene expression in patients' samples after conditioning was not due to different graft cells, but to the conditioning regime. It may also suggested that the patients' diseases did not alter the expression of the analyzed genes, although this has to be confirmed in further studies, because all patients with different diseases were analyzed together that may led to a reciprocal influence on the gene expressions. Both $CD4^+$ and $CD8^+$ T cells reached the same gene expression levels of all analyzed genes 6 months after HSCT as at pre-conditioning state, suggesting that the stem cells engrafted and were capable of expressing genes at the same amount as healthy donor controls.

The conditioning regime led to decreased gene expression of Foxp3, IFN-γ, B7-int and ICOS, whereas it led to increased expression of IL-10 and slightly increased expression of IL-18.

The decreased expression levels were due to the conditioning regime that caused the immune system's downregulation, described above, leading therefore to less immunostimulatory gene expressions. IL-10 is an immunosuppressive cytokine and thus was probably not negatively affected through the conditioning regime. Its expression was contrary slightly upregulated after the conditioning. IL-18 is in contrast to IL-10 an inflammatory cytokine, but its expression was also a tad increased after conditioning, although it was generally quite constant. A possible reason for this decreased expression level could be that the conditioning regime could not downregulate the IL-18 expression and because many other immunostimulatory cytokines had a decreased expression, IL-18 may was slightly heightened as a compensative effect.

This may indicated that the conditioning enhanced the expression of anti-inflammatory cytokines, whereas it

downregulated the expression of the pro-inflammatory cytokines IFN-γ and ICOS. A decreased gene expression of B7-int could be due to an earlier migration of the T cells to the epithelium or mucosal sites or due to the conditioning that suppressed the homing through reduced B7-int gene expression. As discussed below, Foxp3 is may not a restrictive regulatory T cell marker, as believed (Allan et al., 2007; Corthay, 2009; Morgan et al., Wang et al., 2007). $Foxp3^+$ T cells could also represent recently activated T cells that got reduced through conditioning.

6.3 Gene Expression Levels in T Cells - with and without ATG

60 out of all 76 patients got ATG treatment, thus the reduced gene expressions of Foxp3, IFN-γ, ICOS and B7-int that had been seen in the overall gene expression levels in $CD4^+$ and $CD8^+$ T cells of ATG patients, respectively, were probably also caused by ATG and not only by the conditioning regime, as believed. Very reduced gene expression levels of Foxp3, IFN-γ, ICOS and B7-int were connected with the administration of ATG, as our results have shown, indicating that T cell-depleted transplants influenced the overall gene expressions. However, even without ATG treatment the expression of IFN-γ was reduced in $CD8^+$ T cells as well as the expression of B7-int in $CD4^+$ T cells after conditioning, indicating that also the conditioning regime influenced the gene expression levels. The levels of IL-10 and IL-18 were enhanced in both $CD4^+$ and $CD8^+$ T cells after conditioning in case of ATG administration. IL-18 hardly increased without ATG. With this results it could be concluded that ATG influenced the gene expression of the analyzed genes more that the conditioning, although not all genes were influenced equally strong. The expression of ICOS, Foxp3 and IL-18 were probably mainly altered through ATG, whereas the levels of IFN-γ, B7-int and IL-10 were also influenced through conditioning.

6.4 Gene Expressions in Donor Grafts - BMSC versus PBSC

The donors' samples delivered different gene expression levels comparing bone marrow with peripheral blood as stem cell sources. Foxp3, FuT7 and B7-int were more expressed and IL-10 was less expressed in PBSC that in BMSC donor grafts in both $CD4^+$ and $CD8^+$ T cells. IFN-γ and IL-18 had similar expression levels in $CD8^+$ T cells of both stem cell sources, whereas IFN-γ was more and IL-18 less expressed in $CD4^+$ T cells deriving from PBSC grafts. The overall expression of ICOS was equal in both stem cell sources.

But those gene expression differences were not seen in the patients' samples. The expressions of the analyzed genes were equal not mattering if the stem cells derived from the bone marrow or peripheral blood. Furthermore, the development of aGVHD was about the same in patients with BMSC and PBSC grafts, 45%

and 52% respectively, as has been seen in other studies (Bensinger et al., 2001; Meisel et al., 2007). It could be speculated that on the one hand the treatment with ATG had influenced the gene expression levels in both stem cell sources, thus maintaining the same gene levels in the different patient groups, transplanted with BMSC or PBSC, and that on the other hand the donor T cells maybe had changed their gene expression features during time as response to their new environment, independent from their origin.
Contrary to our results has the group of Gallardo seen that patients with PBSC as graft develop more frequently GVHD than patients with BMSC, although their overall survival is the same (Gallardo et al., 2009).

6.5 IFN-γ Plasma Level and its Correlation to aGVHD Development

In our study we could see a significant higher IFN-γ protein level by analyzing the plasma in patients without aGVHD at the day of transplantation ($p = 0.009$) and 3 months after transplantation ($p = 0.037$). The IFN-γ levels trended to an elevated level in the case of no aGVHD development at the timepoints of pre-conditioning ($p = 0.083$), 14 days ($p = 0.082$) and 28 days after transplantation ($p = 0.087$). Through analyzing the gene expression levels in $CD4^+$ and $CD8^+$ T cells we could not detect any significant differences or at least a trend between patients with and without aGVHD, thus suggesting that other cells, mainly NK cells were responsible for a diverse IFN-γ production.

The pro-inflammatory cytokine IFN-γ has been seen to inhibit aGVHD in many mouse models (Brok et al., 1993; Murphy et al., 1998; Sykes et al., 1990; Yang et al., 1998, 2002 and 2005) but also in patients' studies (Bogunia-Kubik et al., 2006; Mlynarczewska et al., 2004; Teshima and Ferrara, 2002), which was confirmed in our study. We could see that patients with elevated IFN-γ protein levels at the day of transplantation did not develop aGVHD, whereas patients with low IFN-γ levels got aGVHD, indicating that low IFN-γ levels at the day of transplantation were associated with a higher probability of aGVHD development.
In murine models different approaches have been performed to identify the role of IFN-γ in the development of aGVHD. It has to be pointed out, that the mice have been lethally irradiated before HSCT in most of the studies. IFN-γ knock out mice have commonly been used as donors or as recipients to see if the disease could occur in absence of this cytokine. It has been approved in diverse studies that aGVHD occurs significantly more often in recipients that get stem cells transplanted from IFN-γ deficient donors, independent of the fact if the recipients are IFN-γ deficient or not (Murphy et al., 1998; Yang et al., 2002 and 2005). This indicates that donor derived and not recipient derived IFN-γ is crucial for the cytokine's protective effect against aGVHD development (Murphy et al., 1998; Welniak et al., 2000; Yang et al., 2005). However recipient derived IFN-γ is seen to be important for a positive transplantation outcome in another way namely by enhancing graft survival (Murphy et al., 1998). Murine studies have also shown that donor derived IFN-γ is important for the positive GVL effect (Sykes et al., 1988; Yang et al., 2002). A further approach for studying the features

of IFN-γ has been the injection of neutralizing anti-IFN-γ mononuclear antibodies or different cytokines into mice. IL-12 administration has been seen to have a protective effect against the development of aGVHD, but if IFN-γ is neutralized, IL-12 looses its ability indicating that aGVHD inhibition through IL-12 is IFN-γ dependent (Murphy et al., 1998; Yang et al., 1998 and 2005). If IL-10 that inhibites a T_H1 response and IFN-γ production is administered to mice recipients after allogeneic HSCT, no amelioration of aGVHD can be observed (Krenger et al., 1994). These findings suggest that T_H1 cytokines, mainly IFN-γ, are preventing the development of aGVHD.

There are different explanations through which mechanisms IFN-γ can lower the appearance of aGVHD after HSCT in humans, some have already been verified in the mouse model.

High IFN-γ levels may deactivate donor T cells or induce activation-induced cell death (AICD) in those lymphocytes after HSCT as it has been described in murin models (Dey et al., 1998; Teshima and Ferrara, 2002). There is evidence that IL-12 induces a strong and rapid *"IFN-γ-dependent AICD of donor T cells" (Teshima and Ferrara, 2002)*, and thus inhibiting the development of aGVHD in mice (Dey et al., 1998; Reddy et al., 2001; Teshima and Ferrara, 2002). AICD may occur due to the IFN-γ-induced Fas expression on donor T cells leading to their caspase8-dependent apoptosis (Dey et al., 1998; Teshima and Ferrara, 2002; Yang et al., 2002 and 2005). The pro-inflammatory cytokine also accelerates Fas and HLA I expression on tumor cells leading to an enhanced GVL effect, thus lowering the risk for relapse (Böhm et al., 1998; Sayers et al., 1998). The Fas-dependent apoptosis of alloreactive donor T cells is one way how IFN-γ is believed to suppress the development of aGVHD, whereas the maintenance of the $P27^{Kip1}$ capacity by IFN-γ is another explanation for the suppression of aGVHD by this T_H1 cytokine. $P27^{Kip1}$ restrains T cell growth and thus inhibits donor T cell proliferation and it is additionally a potential tumor suppressor protein, seen in mice, thus may positively affeccting the GVL effect in HSCT patients. $P27^{Kip1}$ can be downregulated by distinct growth factors, which is prevented by IFN-γ (Takami et al., 2002). Furthermore IFN-γ can inhibit an overproliferation of alloreactive donor T cells and thus prevent aGVHD by upregulating the expression of programmed death ligand 1 (PD-L1) on those lymphocytes (Murphy et al., 1998; Welniak et al., 2007; Yang et al., 2005).

There is evidence that IFN-γ is crucial for homing of alloreactive T cells leading to their migration into lymphoid tissue, where the donor T cells can start an immune reaction against lymphohematopoietic tissue, thus augmenting the GVL effect, whereas IFN-γ is suppressing the donor T lymphocyte migration to *"parenchymal GVHD target tissues" (Yang et al., 2002)*, thus inhibiting aGVHD (Klimpel et al., 1990; Parfrey et al., 1999). Another explanation for the aGVHD preventing feature of IFN-γ is that the cytokine has been seen to be crucial for the development of T_{reg}s in mouse models, whereas it *"inhibites self-antigen-induced generation and activation of $CD4^+CD25^+$ T_{reg}s" (Yang et al., 2005)* (Murphy et al., 1998; Nishikawa et al., 2005; Sawitzki et al., 2005). Those T_{reg}s could prevent the expansion and alloreaction of donor T cells.

The enzyme indoleamine 2,3-dioxygenase (IDO) may also play a crucial role in IFN-γ induced donor T cell deactivation. IFN-γ activates IDO, which consumes tryptophan and suppresses adaptive T cell-mediated immunity, shown in humans, and probably induces therefore tolerance in alloimmunity and transplantation

(Brandacher et al., 2008; Chen et al., 2008). This confirms that high IFN-γ levels decrease the risk of aGVHD development.

Different IFN-γ polymorphisms are associated with inter-individual diverse cytokine production levels, thus are correlated with differing aGVHD outcomes in humans. There are 5 different IFN-γIntron1 alleles, whereas the alleles 2 and 3 are most common and the alleles 4 or 5 are quite rare. Patients, who are homozygous for the IFN-γIntron1 allele 3, which is responsible for lower IFN-γ levels, are more prone to develop aGVHD (Bogunia-Kubik et al., 2006; Cavet et al., 2001). The allele 2 is in contrast associated with higher IFN-γ production and patients being homozygous for this allele develop less common aGVHD or cGVHD (Bogunia-Kubik et al., 2006; Cavet et al., 2001; Pravica et al., 1999). cGVHD has similarities to autoimmune diseases, and IFN-γ has also been postulated to attenuate the outcome of autoimmunity in mice (Caspi et al., 1994; Ferber et al., 1996; Rosloniec et al., 2002; Segal et al., 1998). The appearance of the homozygous IFN-γIntron1 allele 3 has the same aGVHD outcomes as if the homozygous allele 2 is absent, suggesting that the other allele combinations cannot lead to equally high IFN-γ productions as allele 2 (Bogunia-Kubik et al., 2006). The differing nucleotide sequences in those alleles *"may affect the binding of NF-κB transcriptional factor and thus the cytokine production" (Bogunia-Kubik et al., 2006)*. The correlation of high IFN-γ levels and low aGVHD risk could be confirmed in our studies.

But also contrary results have been documented in different murin (Allen et al., 1993; Mowat, 1989; Welniak et al., 2000) and patient studies (Krenger et al., 1997; Holler, 2002; Niederwieser et al., 1990), where a high IFN-γ level has been associated with an augmented GVHD outcome, that is converse to our results. In some mouse models reduced aGVHD appearance is due to the blockade of IFN-γ (Allen et al., 1993; Krenger et al., 1997), and T_H1 cytokines (like IFN-γ and IL-2) are seen to be crucial for the induction of aGVHD (Ferrara et al., 1999; Krenger and Ferrara, 1996; Williamson et al., 1997). Also in preclinical and clinical studies elevated levels of the pro-inflammatory cytokines IL-2 and IL-12, an IFN-γ inducer, have been linked to more aGVHD and especially cGVHD development (Bunjes et al. 1995; Murphy et al., 1998).

The main explanations for a direct correlation of IFN-γ levels and GVHD development are that IFN-γ can activate macrophages to produce pro-inflammatory cytokines (like TNF-α and IL-1), and induce the production of NO (Allen et al., 1993; Niederwieser et al., 1990; Teshima and Ferrara, 2002). Furthermore, T_H1 cytokines are associated with cell-mediated immune reaction, thus augmenting an alloreactive donor T cell response against the recipient as well as the infiltration of these cells into the tissue leading to more GVHD (Allen et al., 1993; Krenger and Ferrara, 1996; Mowat, 1989; Williamson et al., 1997). As described in the positive effects of IFN-γ the cytokine can induce Fas expression on donor T cells, but some murine studies have indicated that it can also upregulate Fas on recipient cells leading to apoptosis of the body's own cells and thus to more severe GVHD (Welniak et al., 2007).

Maybe the different observations of IFN-γ levels and GVHD development do not have to be contradictory to

each other regarding to the point of time when the cytokine is expressed. We could see that only high IFN-γ plasma levels at the day of transplantation were connected to decrease the risk for aGVHD, whereas no high significant correlations between the IFN-γ levels in the plasma and aGVHD outcome could be observed at any other timepoint of sampling, except 3 month after HSCT, although this significance could have occurred coincidentally.

In the murine model an *"early T_H1 polarization of donor T cells by administration of cytokines such as IFN-γ, IL-2, IL-12 and IL-18" (Reddy and Ferrara, 2003)* can decrease aGVHD, suggesting that high IFN-γ levels are important directly after HSCT to attenuate aGVHD (Brok et al., 1993; Reddy and Ferrara, 2003). Maybe later IFN-γ distributions or administrations would be connected with more severe aGVHD or cGVHD. However, this has to be determined in further studies.

The conditioning regime of the patients may be very important concerning the IFN-γ feature to ameliorate GVHD (Murphy et al., 1998; Yang et al., 1998). It is still not fully understood if *"the conditioning can alter the role of IFN-γ" (Murphy et al., 1998)*. Patients treated with fludarabine, an immunosuppressive chemotherapy drug, are producing less IFN-γ thus may have higher risk for getting GVHD (Bogunia-Kubik et al., 2006). Additionally the grade of irradiation has altered the effect of IFN-γ in murine studies. If the recipient gets lethally-irradiated, high IFN-γ levels are more likely seen to protect from aGVHD development, whereas the cytokine can have a deleterious effect in sublethally-irradiated or non-irradiated mice (Murphy et al., 1998; Welniak et al., 2000; Yang et al., 1998). IFN-γ is suggested to attenuate aGVHD in mice after non-myeloablative or myeloablative conditioning (Welniak et al., 2007; Yang et al., 2002 and 2005).

Low donor IFN-γ levels but maybe also patient IFN-γ levels on the verge of transplantation can be postulated as an additional risk factor for the development of GVHD. Different theoretical possibilities are considered for decreasing this risk factor. First of all a donor that is not homozygous for the IFN-γIntron1 allele 3 should be chosen, if possible. IFN-γ and IL-12, that induces the IFN-γ production, could be applied to the patient some days before transplantation to augment the IFN-γ level. Another approach could be to stimulate the donor T cells and/or NK cells in vitro to produce more IFN-γ before the transplantation. This could have the advantage over the strategy of IFN-γ or IL-12 administration that IFN-γ is produced whereto the donor T and NK cells migrate, whereas it is not clear whereto IFN-γ would home after administration, and thus maybe not having the same advantage effect. But these strategies have to be proven in preclinical and clinical trails first. With the knowledge that low IFN-γ plasma levels at the day of transplantation were connected with more aGVHD development the treatment against aGVHD could be started much earlier, which gains time.

6.6 IL-18 Plasma Level at aGVHD Onset

On the day of aGVHD onset IL-18 was significantly increased ($p < 0.001$) in patients, who developed the

disease, comparing to IL-18 plasma levels before aGVHD development, after the onset and to patients without aGVHD in our study. Analyzing the gene expression levels in $CD4^+$ T cells gave a significant higher IL-18 level in patients with aGVHD 21 days after transplantation ($p = 0.008$). The median for aGVHD onset lay 30 days after transplantation leading to a correlation between IL-18 increase and aGVHD onset also in the gene expression level in $CD4^+$ T cells, thus confirmed the correlation of elevated protein plasma level and aGVHD onset. The protein level of IL-12 was not increased at the day of GVHD development, thus probably leading to a T_H2 cell response that is provided by IL-18 without the combination of IL-12 (Kashiwamura et al., 2002; Nakanishi et al., 2001).

Previous studies in mice and humans have detected increased IL-18 levels, if aGVHD developed, but it is debated, if IL-18 causes aGVHD or if its augmentation is a consequence of the disease (Arnold et al., 2002; Fujimori et al., 2000; Min et al., 2004; Nakamura et al., 2000; Reddy et al., 2001; Zecchina et al., 2001). In a patient study IL-18 is not altered due to conditioning or bacterial infections, but augmented IL-18 plasma levels have been observed in the recovery phase, at the time of engraftment, in case of aGVHD development, as supported in our study (Fujimori et al., 2000). To prove if IL-18 causes aGVHD, neutralization antibodies against this cytokine have been used in mouse model leading to the result that blocking of IL-18 does not prevent aGVHD development, whereas the donor T cell expansion and alloreactive CTL activity have not been inhibited. This indicates that IL-18 is not inducing aGVHD, but instead caused by it – at least in mice (Arnold et al., 2002; Reddy et al., 2001). Further clinical studies have to prove this in humans. IL-18 can be involved in tissue repair and regeneration, thus its augmented level is maybe the response to systemic alloreaction appearing in GVHD (Arnold et al., 2002; Kashiwamura et al., 2002; Reddy et al., 2001).

The levels of Akt, a protein, that causes cell survival and inhibits apoptosis and growth arrest and DNA damage-45 (GADD45) that is involved in DNA repair, suppression of cell growth and also cell survival can be elevated by IL-18, seen in mice and humans (Min et al., 2004; Yang et al. 2001). Akt and GADD45 may are activated after aGVHD by IL-18 leading to tissue repair and thus having a positive effect on the host but they can also inhibit apoptosis, which would support the donor T cells to expand and thus also having a negative effect in combating aGVHD.

Although IL-18 is also attributed to a protective effect against aGVHD in mice, this is skeptical, because this positive effect failes to appear in absence of IFN-γ, indicating that IFN-γ and not IL-18 is mainly responsible for decreasing aGVHD development. If IL-18 has been administered early after HSCT in mouse studies, a decline of aGVHD is detected due to reduced donor T cell expansion caused by elevated Fas expression levels and thus more apoptosis of alloreactive donor T cells (Min et al., 2004; Reddy et al., 2001 and 2003). This Fas-dependant apoptosis is also IFN-γ regulated and connected with a T_H1 response, thus this is no evidence for a protective effect of IL-18 against aGVHD (Arnold et al., 2002; Reddy et al., 2001 and 2003).

It has been seen in mice that the expression of CD25 and CD69 are upregulated on donor T cells through IL-18, thus leading to more activated donor T cells and worsening aGVHD (Reddy et al., 2001). But cGVHD can

in contrast be ameliorated through the cytokine *"by promoting the development of regulatory alloreactive CTLs" (Arnold et al., 2002)*, as seen in mice and humans (Min et al., 2004; Okamoto et al., 2000).
IL-18 has been seen to influence T_H polarization and other cytokine responses differently *"depending on weather the donor T cells are resting or already activated" (Reddy et al., 2003)*, thus leading to different effects on GVHD. The GVL effect can also be preserved in presence of IL-18, as proven in different studies (Min et al., 2004; Reddy et al., 2003).
The grade of aGVHD has been shown to positively correlate with the IL-18 plasma level and after a successful aGVHD treatment the IL-18 level decreases again (Arnold et al., 2002; Fujimori et al., 2000). This indicates that IL-18 may be a good marker for diagnosing aGVHD, which would help to start an early treatment of the disease. To be able to determine the onset day of aGVHD that was correlated with augmented IL-18 plasma levels in our study, would be a big step forward in diagnosing aGVHD (especially at grade I and II) right, and thus gaining time in its treatment. Regarding the current results the advantage of IL-18 is that the cytokine can be used as a marker for aGVHD, but not as a regulative cytokine that could be applied in therapy for ameliorating aGVHD.

6.7 Foxp3 Gene Expression in $CD8^+$ T Cells in Case of aGVHD

We could observe significantly elevated Foxp3 gene expression levels in $CD8^+$ T cells at the day of transplantation ($p = 0.005$) and 28 days after HSCT ($p = 0.020$) in patients with aGVHD. 21 days ($p = 0.083$) and 6 months ($p = 0.053$) after transplantation a trend could be reported towards increased Foxp3 gene expression levels in $CD8^+$ T cells of aGVHD patients. Although a significant Foxp3 gene expression increase was detected in $CD4^+$ T cells 21 days after HSCT ($p = 0.016$) in patients with aGVHD, this statistical significance might occurred by chance, because no trend of augmented Foxp3 levels had been seen at any other timepoints as in $CD8^+$ T cells. These results were unexpected, because the transcription factor Foxp3 is seen to be a specific marker for T_{reg}s, which can induce tolerance and prevent inflammatory and autoimmune diseases, thus were believed to ameliorate aGVHD, as detected in other studies (Chen et al., 2007; Mielke et al., 2007; Miura et al., 2004; Pallandre et al., 2007; Rezvani et al., 2006).
We only measured the gene expression hence the mRNA level and not the protein level of Foxp3 in T cells. Because Foxp3 is intracellular, its protein level could be analyzed with FACS after fixation and permeabilization of the cells to see if the Foxp3 mRNA level would correlate with the protein level. This was done in another human study confirming a correlation between its mRNA and its protein level, so that it could be suggested in our study that the Foxp3 mRNA reflected its protein level thus the $Foxp3^+$ $CD8^+$ T cells could be seen as certain $Foxp3^+$ (Rezvani et al., 2006).

In diverse mouse and patient studies elevated Foxp3 gene expression levels have been connected with a lower

aGVHD outcome, in contrast to our results. Although most of the studies have determined T_{reg}s by analyzing Foxp3 expression in $CD4^+$ T cells and not in $CD8^+$ T cells, considering that only little is known about $CD8^+$ T_{reg}s. These studies suggeste that the $CD4^+$ $Foxp3^+$ T_{reg}s lead to immunosuppression and thus prevent aGVHD (Mielke et al., 2007; Miura et al., 2004; Pallandre et al., 2007; Rezvani et al., 2006; Rieger et al., 2006). It has been seen that high donor and not recipient T cell rates, which are $CD4^+$ and $Foxp3^+$ are essential for the ameliorating effect on aGVHD in humans, even if the transplant is T cell-depleted (Rezvani et al., 2006). The gene expression level of Foxp3 in $CD4^+$ T cells is reduced in patients with aGVHD comparing to a healthy population, whereas it is increased in patients without aGVHD (Mielke et al., 2007; Miura et al., 2004; Rezvani et al., 2006; Rieger et al., 2006). Besides, the number of appearing $Foxp3^+$ T_{reg}s can be linked to the occurring grade of GVHD in humans (Miura et al., 2004).

In order that T_{reg}s can act in a suppressive way a cell-cell contact between the T_{reg} and the suppressed T cell is necessary (Cosmi et al., 2003; Lee et al., 2005; Takahashi et al., 1998). Thus $Foxp3^+$ T_{reg}s may have to be present in target tissues of aGVHD to can prevent the development of the disease by suppressing alloreactive T cells there. It has been detected in mouse model that $CD4^+$ $Foxp3^+$ T cells are decreased in aGVHD target tissues during the disease, whereas those T cell levels are elevated, if no aGVHD occurs (Chen et al., 2007). The T_{reg}s level in the peripheral blood does not have to reflect their level in GVHD target tissues (Rieger et al., 2006). This could indicate that, as we could see in our study, elevated $Foxp3^+$ T cell levels in the peripheral blood may were connected with reduced $Foxp3^+$ T cell levels in aGVHD target tissues, like the skin, mucosa or lung, thus leading to more aGVHD. If no aGVHD occurred in the patients, the regulatory $Foxp3^+$ T cells may had already migrated to the target tissues, where they could suppress the function of alloreactive T cells correlating with reduced $Foxp3^+$ $CD8^+$ T cells in the peripheral blood, like measured in our study.

It has been seen in human studies that the chemokine receptor CCR4 is important to trigger T_{reg}s into skin and lungs, thus $Foxp3^+$ T cells that maintain in the blood are maybe $CCR4^-$ that would explain why some $Foxp3^+$ T cells migrate to aGVHD target tissues ($CCR4^+$) and others (CCR^-) do not (Fontenot et al., 2003; Lee et al., 2005; Sather et al., 2007).

It is still not fully understood, if a distinct T_{reg} lineage does exist, or if the T_{reg}s that are known to have suppressive characteristics also have activating features, as well as effector T cells can have suppressive effects too (Corthay, 2009). Furthermore, Foxp3 as specific marker for T_{reg}s is challenged, because it has been detected that *"human $CD4^+$ and $CD8^+$ T cells transiently express Foxp3 upon activation" (Corthay, 2009)* (Morgan et al., 2005; Wang et al., 2007). Other markers that have been used in different studies to identify T_{reg}s, like CD25, CTLA-4 or glucocorticoid-induced tumor necrosis factor receptor (GITR) were not exclusively expressed on T_{reg}s either. Thus no specific marker for T_{reg}s is known yet. This suggests that the $CD8^+$ $Foxp3^+$ T cells, which we measured, are not T_{reg}s without any doubt. There is the assumption that $Foxp3^+$ T cells are not totally differentiated T_H cells (Corthay, 2009). A human study indicates that *"de novo T cell development" (Miura et al., 2004)* is correlated with Foxp3 gene expression (Miura et al., 2004). It is assumed that *"Foxp3 regulatory T cells may differentiate in vivo into conventional effector T_H cells, with or without concomitant downregulation of Foxp3." (Corthay, 2009).* Thus, in our study the $Foxp3^+$ $CD8^+$ T cells

could be recently activated T cells explaining that more Foxp3 gene expression in those probably effector $CD8^+$ T cells, hence alloreactive T cells against the host, was connected with more aGVHD. However, this did not explain why $CD4^+$ T cells of patients with aGVHD did not express more Foxp3 than patients without aGVHD in our study, except at one timepoint (21 days after HSCT), which may was by chance. One possible explication could be that only $Foxp3^+$ $CD8^+$ T cells and not $Foxp3^+$ $CD4^+$ T cells could react as alloreactive T cells in case of aGVHD, or that aGVHD was mostly $CD8^+$ T cell-mediated. Very little is known about $Foxp3^+$ $CD8^+$ T cells, hence these opinions have to be verified in further experimental and clinical trails.

6.8 ICOS Gene Expression and its Correlation to aGVHD Development

In our study the correlation between ICOS gene expression levels in both $CD4^+$ and $CD8^+$ T cells at day 21 ($p = 0.029$ and 0.038 respectively) suggested that decreased ICOS levels were connected with reduced GVHD development. This indicated that low ICOS levels on the verge of GVHD onset (median of onset 30 days after HSCT) were important for the prevention of the disease.

Most of the studies have been done in mice and not human regarding ICOS and the outcome of GVHD after HSCT. Those results cannot be adopted one-to-one to humans, but they give a hint which role ICOS play in human GVHD development. Different studies have observed that the blockade of ICOS by either using ICOS deficient mice as donors or recipients or anti-ICOS mononuclear antibodies lead to reduced GVHD (Hubbard et al., 2005; Taylor et al., 2005; Yu et al., 2006). Furthermore alloreactive donor T cells express more ICOS during GVHD in mice, confirming our results (Hubbard et al., 2005). This is regardless of whether the alloresponse is $CD4^+$ or $CD8^+$ mediated (Taylor et al., 2005). There are varying debated mechanisms how ICOS suppresses an alloresponse in case of GVHD. It has been detected in mouse models that the number of effector T celles is reduced in *"some lymphoid organs and GVHD target organs early after transplantation" (Taylor et al., 2005)*, thus effecting T cell migration and homing, but not their activation, if the costimulatory molecule is blocked. Other results indicate that alloreactive effector T cells are inhibited or that T cells are turned to T_H2 differentiation, without altering their activation, proliferation, homing or cytotoxicity in case of ICOS blockade (Hubbard et al., 2005; Yu et al., 2006). However, in mice also impaired T cell proliferation caused by ICOS deficiency has been observed due to decreased IL-2 production after activation (Dong et al., 2001; Taylor et al., 2005). If reduced ICOS expression is connected with less IL-2 production leading to decreased GVHD development in patients have to be studied further in clinical trials. In case of a correlation between ICOS and IL-2 expression, blocking the costimulatory molecule may be a good approach in GVHD treatment. But also a reduced expression of FasL has been associated with ICOS deficiency in murine models leading to reduced effector functions of alloreactive donor T cells as well as less GVHD (Yu et al., 2006).
Also converse results have been received from different groups, where ICOS blocking has been seen to

exacerbate aGVHD and reduce only cGVHD in mice. This has been observed in non-irradiated as well as in sublethally irradiated mice (Ogawa et al., 2001; Taylor et al., 2005; Yu et al., 2006). If ICOS costimulates T cell response is probably affected by *"the nature of immune response, such as the strength of the TCR signal, involvement of innate immunity, and CD28 costimulation" (Yu et al., 2006).* A strong antigen recognition may lead to a costimulation of CD28 and involvement of the innate immunity, thus to more AICD through ICOS leading to a negative correlation between ICOS expression and GVHD development, whereas a weak antigen recognition may does not involve the innate immunity or CD28 costimulation leading to enhanced activity of the T cells and accelerated GVHD (Wallin et al., 2001; Yu et al., 2006).

Our results that higher ICOS gene expression levels were connected with the development of GVHD have to be confirmed in further clinical studies. It is also of interest if the gene expression levels are correlating with the protein levels, which could be done by FACS analysis. ICOS probably does not always accelerate GVHD depending on the strength of alloantigen recognition and maybe IL-2 plays a central role in ICOS-mediated GVHD development.

6.9 FuT7 Gene Expression in $CD8^+$ T Cells in Case of aGVHD

FuT7 expression plays an important role in the onset of an inflammatory response leading to leukocytes' homing to sites of infection by upregulating the expression of E- and P-selectin ligands and L-selectin on leukocytes (Bengtson et al., 2002; Dudda et al., 2008; Martinez et al., 2005). We detected a significant correlation between decreased FuT7 gene expression in $CD8^+$ T cells in the peripheral blood and the development of aGVHD 21 days ($p = 0.001$) and 28 days ($p = 0.023$) after HSCT.

Murine studies have shown that the interaction between selectins and their ligands are crucial for GVHD development so that the alloreactive donor T cells can role on the endothelial cells and migrate to mucosal lymphoid tissues (Dutt et al., 2005; Li et al., 2004; Petrovic et al., 2004). In a GVHD mouse model it has been detected that P-selectin ligand levels are increased, whereas L-selectin levels are decreased on activated $CD8^+$ T cells in case of GVHD (Asaduzzaman et al., 2009). Those cells have been measured in GVHD target tissues and not in the peripheral blood as in our study. This could suggest that a lower FuT7 gene expression in $CD8^+$ T cells in the peripheral blood may be connected with increased GVHD development, as we could show, because the alloreactive $CD8^+$ $FuT7^+$ T cells have already migrated to GVHD target tissues. As our results indicated the $CD8^+$ $FuT7^+$ T cells remained in the blood, if no aGVHD occurred that was possible due to a loss of E-selectin and P-selectin expression on the endothelium thus prohibiting leukocyte rolling.
Other clinical studies have shown that serum levels of soluble E-selectin are elevated in patients, who develop aGVHD (grade II – IV) around aGVHD onset (day 30), as well as E-selectin expression on endothelial cells is

connected with aGVHD (Matsuda et al., 2001; Norton et al., 1992 and 1993). This proves the theory that $CD8^+$ $FuT7^+$ T cells remains in the blood without aGVHD due to reduced E-selectin expression. Activation and damage of the endothelium are seen to be connected with the appearance of aGVHD and also cGVHD (Matsuda et al., 2001).

Elevated E-selectin expression levels on endothelial cells are probably as well as a decreased P- and E-selectin ligand expressions on $CD8^+$ T cells in the peripheral blood, indicated by reduced FuT7 gene expression, good markers for the onset of aGVHD, whereas it is supposable not that easy to engage in this mechanism for treating GVHD. As detected in murine studies E-selectin *"inhibition has no effect on GVHD-induced $CD8^+$ T cell endothelium interactions" (Asaduzzaman et al., 2009)* and alloreactive donor T cells can migrate to GVHD target tissues *"independently of P- and E-selectin ligand" (Eom et al., 2005)*. Thus FuT7 may be used as molecular marker for diagnosing aGVHD.

6.10 Hypothesis about $CD8^+$ T cell-mediated aGVHD

The interpretation of our results led to the hypothesis that aGVHD was mainly mediated by $CD8^+$ T cells. Concerning that $CD8^+$ $FuT7^+$ but not $CD4^+$ $FuT7^+$ T cells were recruited to GVHD target tissues in case of aGVHD probably indicated that the disease was $CD8^+$-mediated. This could be fortified regarding that elevated $CD8^+$ $Foxp3^+$ T cells, seen as recently activated T cells, in the peripheral blood were correlating with increased aGVHD development. However, if the $CD8^+$ $Foxp3^+$ T cells would be seen as T_{reg}s, the $CD8^+$ T cells were maybe not only mediating aGVHD development, but also suppressing it, because less $CD8^+$ $Foxp3^+$ T cells in the peripheral blood, as it was detected in case of aGVHD, would be seen to be connected with more $CD8^+$ $Foxp3^+$ T cells in GVHD target tissues, where they could suppress alloreactive responses.

No such correlations between aGVHD development and $CD4^+$ T cell gene expressions could be detected in our studies, besides elevated IL-18 levels at day 21 in case of aGVHD, which was probably the consequence of aGVHD and not causing the disease.

6.11 Outlook

Further studies have do be done to confirm our results and before the gained knowledge can be implemented in GVHD prophylaxis and diagnosis. The administration of IFN-γ or IL-12 has to be tested in clinical trials to see if they ameliorate the outcome of GVHD before they can be used as prophylaxis against the disease. It is important to be conscious of the fact that cytokines can influence, compensate or suppress each other. Thus a cytokine can act differently in diverse cytokine surroundings. Furthermore, different cytokine gene polymorphisms can lead to variable cytokine productions. This is crucial to think of and test, if any cytokine-dependent therapeutic setting is perfomed to avoid the outcome of contrary results.

7 Conclusions

- Higher IFN-γ plasma levels (> 200 pg/ml) at the day of HSCT are connected with a reduced risk for aGVHD development. Thus low IFN-γ plasma levels at the day of HSCT are a molecular marker for aGVHD.
- The application of IFN-γ or IL-12 immediately before HSCT, or an in vitro stimulation of NK or T cells before HSCT to produce more IFN-γ could probably lower the occurrence of aGVHD.

- Elevated IL-18 plasma levels at the day of aGVHD onset could be used as a marker for diagnosing aGVHD leading to an early therapy initiation.
- Higher Foxp3 gene expressions in $CD8^+$ T cells at the day of HSCT as well as 28 days after transplantation in case of aGVHD could indicate the disease development.
- FuT7 may be a capable diagnostic marker for aGVHD as well, because its gene expression is enhanced in $CD8^+$ T cells 21 and 28 days after HSCT in case of aGVHD.
- ICOS may also be a possible molecular diagnostic marker for aGVHD, because its gene expression is enhanced in $CD4^+$ and $CD8^+$ T cells in aGVHD patients 21 days after HSCT.

- Gene expression levels in $CD4^+$ and $CD8^+$ T cells are altered through ATG administration. The expression levels of Foxp3, IFN-γ, ICOS and B7-int are mainly affected.
- Foxp3, IFN-γ, IL-10, IL-18, ICOS, FuT7 and B7-int gene expression levels of donor grafts differ between stem cells deriving from bone marrow and from peripheral blood. However, no significant gene expression differences occur in patients with diverse stem cell grafts.

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9 List of abbreviations

aGVHD	acute GVHD
AICD	Activation-induced cell death
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
AP-1	Activator protein-1
APC	Antigen-presenting cell
ATG	Antithymocyte globulins
Bcl-2	B cell lymphoma-2
BMSC	Bone marrow stem cells
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CBSC	Cord blood stem cells
CCR	Chemokine (cystein-cystein motif) receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
cGVHD	Chronic GVHD
CLA	Cutaneous lymphocyte antigen
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CR	Complete remission
CS-1/FN	Connecting segment-1 region of fibronectin
C_t	Threshold cycle
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
CVID	Common variable immunodeficiency
DBY	Dead box RNA helicase Y
DFFRY	Drosophila fat-facets related Y gene
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide-triphosphate
DTT	Dithiothreidol
dTTP	Deoxythymidin-triphosphate
dUTP	Deoxyuridine-triphosphate
ELISA	Enzyme-linked immunosorbent assay
ESL-1	E-selectin ligand-1
FAM	6-carboxy fluorescein

FGF-7	Fibroblast growth factor-7
Foxp3	Forkhead box P3
FuT	Fucosyltransferase
GADD45	Growth arrest and DNA damage-45
GALT	Gut-associated lymphoid tissues
G-CSF	Granulocyte colony-stimulating factor
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
G6PD	Glucose-6-phosphate dehydrogenase
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
GVT	Graft-versus-tumor
HCl	Hydrogen chloride
HEVs	High endothelial venules
HLA	Human leukocyte antigens
HPE	High performance ELISA
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
H_2SO_4	Sulfuric acid
HSV	Herpes simplex virus
HVG	Host-versus-graft
ICOS	Inducible costimulator
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon
IFNR	Interferon receptor
Ig	Immunoglobulin
IL	Interleukin
Int	Integrin
IRF-1	Interferon regulatory factor-1
JAK	Janus kinase
KCl	Potassium chloride
KGF	Keratinocyte growth factor
KIR	Killer immunoglobulin-like receptors
LPAM-1	Lymphocyte Peyer's patch adhesion molecule-1
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MAPKinase	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MDS	Myelodisplastic syndromes

$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MiHAs	Minor histocompatibility antigens
MMF	Mycophenolate mofetil
mRNA	Messenger RNA
MUD	Matched unrelated donor
MyD88	Myeloid differentiation primary response gene 88
NaCl	Sodium chloride
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NO	Nitric oxide
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cells
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1	Programmed deat-ligand 1
pdN_6	Random hexamer primer
PGE_2	Prostaglandin E2
Ph	Philadelphia
PSGL-1	P-selectin glycoprotein ligand-1
RIC	Reduced-intensity conditioning
RNA	Ribonucleic acid
ROR	Retinoic acid-related orphan receptor
Rpm	Rounds per minute
RQ-PCR	Real time quantitative-PCR
RT	Reverse Transcriptase
SFN	Scurfin
sLe^x	Sialyl Lewis x
STAT	Signal transducers and activators of transcription
TAMRA	6-carboxy-tetramethyl rhodamine
T-bet	T-box, expressed in T cells
TBI	Total body irradiation
TCR	T cell receptor
TGF	Transforming growth factor
T_H	T helper cell
TLR	Toll-like receptor
TNF	Tumor nicrosis factor
TRAF6	TNF receptor-associated factor 6

TRAIL	TNF-related apoptosis-inducing ligand
TRM	Transplantation-related mortality
T_{reg}	Regulatory T cell
Tyk	Tyrosine kinase
UCB	Umbilical cord blood
UNG	Uracil-DNA-glycosylase
UTY	Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked
VCAM-1	Vascular cell adhesion molecule-1
ZAP70	Zeta-chain-associated protein kinase 70

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11 Cirrculum vitae

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2004 – 2007:	Study of Genetics with Immunology as major study subject at the University of Vienna
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12 Zusammenfassung auf Deutsch

Die Transplantation von hämatopoetischen Stammzellen (HSCT) ist eine häufig angewandte Therapieform bei Patienten mit blutbildenden Störungen, bei denen konventionelle Chemotherapie nicht durchgeführt werden konnte bzw. erfolglos war. Die Hauptkomplikation nach HSCT ist neben Rezidiv (Rückfall der Krankheit) graft-versus-host Reaktion (GVHD), bei der immunokompetente Donatorzellen Wirtszellen als fremd erkennen können und eine Immunantwort gegen den Wirt starten. Diese Erkennung erfolgt durch Unterschiede in den humanen Leukozytenantigenen (HLA) zwischen Donator und Wirt. GVHD führt zu Gewebeschäden, die zuerst den Thymus, sekundäre lymphoide Gewebe, die Haut, Leber und den Verdauungstrakt schädigen, aber auch zum Tod führen kann. Es ist daher von großem Interesse GVHD so früh wie möglich zu diagnostizieren um rasch mit einer Behandlung beginnen zu können und den weiteren Verlauf der Reaktion zu unterbinden. Heutzutage wird GVHD nur über klinische Symptome diagnostiziert.

Das Ziel unserer Studie ist es molekulare Marker zu finden um GVHD diagnostizieren und prognostizieren zu können bevor Symptome auftreten, sowie den Ausgang von HSCT in weiteren Studien verbessern zu können, indem GVHD durch die Beeinflussung von regulatorischen Molekülen verringert wird.

Bei unserer Studie waren 76 Patienten, die alle mit allogenetischer HSCT behandelt wurden, mit einem Durchschnittsalter von 45 Jahern beteiligt. Leukämie und Myelodysplastisches Syndrom (MDS) waren die Haupterkrankungen der Patienten. Knochenmarksstammzellen (BMSC) und periphere Blutstammzellen (PBSC) waren die am meisten verwendeten Stammzellenquellen für HSCT, wobei 7 Patienten Nabelschnurstammzellen (CBSC) als Transplantat erhielten. Wir analysierten die Genexpressionsniveaus von IFN-γ, IL-10, IL-18, ICOS, Foxp3, FuT7 und B7-int in $CD4^+$ und $CD8^+$ T Zellen vor der HSCT und zu unterschiedlichen Zeitpunkten danach, sowie die Genexpressionsniveaus der Donatorzellen mithilfe von Realzeit quantitativer-PCR (RQ-PCR). Mittels enzyme-linked immunosorbent assay (ELISA) maßen wir die Cytokinproteinniveaus der Donatoren und Patienten (zu unterschiedlichen Zeitpunkten) von IFN-γ, IL-10, IL-18, IL-12, IL-17 und IL-23 im Plasma. Die Genexpressions- und Proteinplasmaniveaus wurden analysiert um eine Korrelation zwischen diesen Niveaus und dem Auftreten von GVHD zu finden.

Schlussfolgernd könnte IFN-γ als molekularer Prognosemarker für eine GVHD-Entwicklung verwendet werden und IL-18, Foxp3, FuT7 sowie ICOS als mögliche Diagnosemarker. Am Tag der HSCT korrelieren höhere IFN-γ Plasmaniveaus (> 200 pg/ml) mit einem verringerten Auftreten von aGVHD ($p = 0.009$) und die Plasmaniveaus von IL-18 sind am Entstehungstag von GVHD erhöht ($p < 0.001$). Die Genexpressionsniveaus von Foxp3 ($p = 0.005 – 0.020$), FuT7 ($p = 0.001 – 0.023$) und ICOS ($p = 0.005 – 0.029$) sind zu unterschiedlichen Zeitpunkten nach der HSCT im Falle von GVHD erhöht.

IFN-γ ist möglicherweise ein Schlüsselcytokin. Durch das Eingreifen in die Produktion von IFN-γ könnte vielleicht das Auftreten von aGVHD verringert werden.