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**Author:** Kim, Yeung-Hyen  
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1. Hematopoietic stem cell transplantation

Hematopoietic stem-cell transplantation (HSCT) is an intensive adoptive cellular immunotherapy for the treatment of hematological malignancies and immune disorders\(^1,2\). The first allogeneic HSCT was successfully performed in 1957. In that year, E. Donnall Thomas initiated the protocol using healthy donor-derived bone marrow (BM) stem cells to treat six patients who suffered from hematological malignancies. The follow-up of these patients showed that the intravenously infused BM cells engrafted and produced new blood cells\(^3\). Between the first BM transplantation and 2006, a total of 50,417 first HSCTs were reported performed worldwide. Of these transplants, 28,901 (57%) were performed with autologous HSCT. The remaining 21,516 (43%) were allogeneic transplants, of which 11,928 were from family donors and 9,588 were from healthy unrelated donors\(^4\). Currently, allogeneic HSCT is also applied for non-malignant disorders such as BM failures, hemoglobinopathies, primary immune deficiencies and inherited disorders of metabolism\(^5\). The use of allogeneic HSCT expanded rapidly. It is, however, still hampered by significant morbidity and mortality. An analysis of the European Blood and Marrow Transplantation (EBMT) data from 1980 to 2001 with a total of 14,403 patients transplanted for early leukemia, acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) in first complete remission or chronic myeloid leukemia (CML) in first chronic phase, show the following causes of death after allogeneic HSCT: graft-versus-host disease (GVHD) (1315 patients; 25% of deaths), infections (597 patients; 11% of deaths caused by, in particular, bacteria (217 patients; 36%), viruses (183 patients; 31%), fungi (166 patients; 28%), or parasites (32 patients; 5%), or ‘other’ causes (1875 patients; 34% of deaths)\(^6\). According to the EBMT report survey in 2011, transplant related mortality (TRM) has decreased from around 50% in 1974-1979 to 20% in 1997-2001. Although the results significantly improved, transplant-related complications after HSCT still remain a major issue.

Most importantly, HSCT facilitates cure of the disease through its graft-versus-leukemia/graft-versus-myeloma (GVL/GVM) effect\(^7-10\), resulting in 20% to 90% complete remissions depending on the malignancies\(^11\); complete remission was observed in 70% to 80% of the patients with CML in chronic phase\(^10,12,13\) in 20% to 35% of all patients with CML in accelerate phase and acute leukemia\(^7,14\), and in 50% of the patients with multiple myeloma\(^15,16\). Allogeneic HSCT can be obtained from three sources: BM\(^17-19\), peripheral blood via
granulocyte-colony stimulating factors (G-CSF) mobilization (PBSC)\textsuperscript{17-20}, and umbilical cord blood (UCB)\textsuperscript{21-24}. In general, BM grafts show lower chronic GVHD, but it causes a slower engraftment, poorer survival in advanced disease and may have a risk for the donor. PBSC grafts show fastest engraftment and better survival in advanced disease. However, it causes more chronic GVHD and more risk for the donor. For adult patients either BM or PBSC are routinely used for the reconstitution of immune system after radiation and/or chemotherapy. Clinical data collected between 2003 and 2006 by the Center for International Blood and Marrow Transplant Research (CIBMTR), demonstrate that 28\% of the transplants in adults and 42\% of transplants in children were performed using BM\textsuperscript{25}, and approximately 70\% and 30\% are done using PBSC grafts in adults and children, respectively, compared with 30\% and 10\% in the late 1990s, respectively\textsuperscript{26}. Despite numerous reports described the choice of stem-cell source, the results are still controversial. Comparing PBSC with BM in pediatric patients, Eapen \textit{et al.} showed a faster engraftment and a survival benefit in advanced disease for PBSC\textsuperscript{26}. A already mentioned above, transplanting PBSC may, however, also lead to a higher rate of chronic GVHD\textsuperscript{26}.

UCB is a well-known source for HSCT and has an effective therapeutic option for patients with hematological malignancies for whom an HLA-identical related donor (IRD) or HLA-matched unrelated donor (MUD) is not available. The clinical results after HLA mismatched UCB show a relatively low incidence and severity of GVHD\textsuperscript{23,24,27}. UCBs contain, however, a low stem-cell dose. This low dose leads to the slow engraftment and to high Transplantation Related Mortality (TRM)\textsuperscript{17,19,21-24}. Nowadays, more than 2,000 unrelated umbilical cord blood transplantations (UCBTs) have been performed\textsuperscript{22,28}. Generally, UCB has been successfully applied in children, but in adults the main difficulties are graft failure and delayed engraftment. Nonetheless, the use of UCBTs in adults has been increasing due to the development of techniques to optimally select cord blood\textsuperscript{22,28-30}.

2. Immunological responses after hematopoietic stem-cell transplantation

2-1. Background
The major problem of HSCT is the immune response of the graft against the recipient i.e. the donor’s immune system attacking the recipient (referred to as “graft-versus-host, GVH”) and the immune response of recipient against the grafted donor cells (referred to as “host-versus-
graft, HVG”). These two immune responses are described in more detail in section 2-2. Importantly, the success of allogeneic HSCT depends on immune cells capable of executing the curative GVL reactions. The crucial role of the latter immune cells in the GVL reactivity has been demonstrated in clinical studies with lymphocyte depleted HSCT. Depletion lead to increased relapse rates up to 40%\(^{31}\). To reduce these problems, additional and alternative strategies are currently being applied, such as donor lymphocyte infusion (DLI).

The use of DLI is effective in patients with relapsed leukemia after allogeneic HSCT. However, DLI enhances the development of GVHD. Moreover, this treatment is most effective for CML patients\(^{32,33}\). The use of DLI and its effects are discussed in section 2-3.

Non-myeloablative allogeneic HSCT (called also as “mini-transplants”) may effectually resemble a standard allogeneic HSCT, but is accompanied by a lower regimen-related toxicity. This strategy can be applied in multiple myeloma patients who’s HSC do not engraft, but they have an autologous recovery due to the non-myeloablative preparatory regimen. However, most non-myeloablative transplants require DLI for maximum GVL effect, thus increasing the risk of GVHD\(^{34}\).

2-2. Graft-versus-host disease

Following HLA-matched allogeneic HSCT, donor T cells may initiate life threatening GVHD. It is well recognized that these donor T cells are directed against disparities beyond HLA, designated as minor Histocompatibility (H) antigens. GVHD can be divided into two types; acute GVHD and chronic GVHD. This distinction is based upon histopathological differences. Both types of GVHD occur in different grades which is related to the severity of the disease.

In HLA identical transplants acute GVHD ranges from 26% to 32%\(^{35}\), leading to a 100-day GVHD-related mortality rates of 20%\(^{36}\). In MUD transplantation, GVHD ranges between 42% to 52%\(^{36}\). The fact that GVHD can be effectively prevented by depletion of mature T cells from stem-cell grafts\(^{37,38}\), underscores the critical role of host reactive donor T cells in GVHD. Generally, these donor T cells target the patient’s foreign minor H antigens\(^{39}\). The role of minor H antigen-specific T cells in GVHD is further discussed in section 4-1.

2-2-1. Acute graft-versus-host disease

Acute GVHD typically occurs within 100 days after allogeneic HSCT. It may involve the skin,
gastrointestinal tract, lung, and/or the liver, and is often fatal. In general, severity of acute GVHD is ascertained by the extent of involvement of the three main target organs: skin, gastrointestinal tract, and liver. Overall grades are I (mild), II (moderate), III (severe), and IV (very severe). Severe acute GVHD has a poor prognosis; 5-years long-term survival for grades III and IV shows 25% and 5%, respectively. Recent clinical studies demonstrated that the median incidence of clinically significant (grade II-IV) acute GVHD is about 40%. However, the incidence of acute GVHD ranges from 10 to 80%. Risk factors are the use of an MUD or a multiparous female donor, older age of the recipient, graft type (PBSC>BMT>CB), and certain conditioning regimens. Moreover, severe acute GVHD (grade III-IV) was observed in 35-45% of recipients of fully matched IRD graft, whereas this was 60-80% for recipients of a one-antigen mismatched MUD graft. The treatment of acute GVHD mainly consists of immunosuppressive prophylaxis, high-dose corticosteroid such as prednisone, but it often leads to deadly infections.

Acute GVHD is mediated by donor effector cells that encounter a foreign environment that have been altered to promote the activation and proliferation of inflammatory cells. Several reports suggest that the complex cellular interactions and inflammatory cascades of acute GVHD can be conceptualized as a five step process: Step 1: Priming of the immune response. Conditioning regimen can induce tissue damage, and host antigen-presenting cells (APCs) are activated by proinflammatory cytokines that lead to donor T-cell amplification. Step 2: T-cell activation and costimulation. Activation may follow as a consequence of interactions between costimulatory molecules of host APCs and donor T-cell receptor (TCR). Step 3: Alloreactive T-cell expansion and differentiation. Step 4: Activated T-cell trafficking. Once activated, T cells migrate to GVHD target organs, where they may recruit other effector leukocytes. Step 5: Destruction of the target tissues by effector T cells.

2-2-2. Chronic graft-versus-host disease

Chronic GVHD generally develops after 100 days of allogeneic HSCT. It is well recognized that chronic GVHD is the primary determinant of late morbidity and mortality after allogeneic HSCT, although it less often results in death. Chronic GVHD may follow acute GVHD, but not all the cases of acute GVHD develop to chronic GVHD. Chronic GVHD may also develop de novo. Nonetheless, the most important risk factor is the previous occurrences of
acute GVHD. Patients who develop secondary chronic GVHD immediately following acute GVHD have the worst prognosis. Thus, the most effective prophylaxis for chronic GVHD is the prevention of acute GVHD\textsuperscript{62}. In addition to inflammation, chronic GVHD may lead to the development of fibrosis, or scar tissue, similar to scleroderma; it may cause functional disability and require prolonged immunosuppressive therapy.

The incidence of chronic GVHD is influenced by the wider availability of PBSC and the increased age of transplant recipients. Overall, the development of chronic GVHD was 30\% in recipients with full IRD graft, opposed to 70\% in patients receiving MUD grafts\textsuperscript{36,37,39,40}.

**2-3. Graft-versus-leukemia effect**

GVH reactivity after allogeneic HSCT may be beneficial for the patient\textsuperscript{63}, as it may help to eradicate residual leukemia cells. This effect is known as the GVL effect. In patients with leukemia and lymphoma, the newly donor-derived immunocompetent cells can recognize the remaining recipient’s malignant cells and destroy them after allogeneic HSCT. This destructive GVL effect reduces the rate of disease relapse\textsuperscript{64,65}.

Earlier studies demonstrate that depletion of T cells from grafts can prevent GVHD\textsuperscript{66}. However, the beneficial effect of T-cell depletion on GVHD was accompanied by an increased relapse rate. The increased relapse rate after T-cell depletion was most obvious in CML patients, followed by AML and ALL patients\textsuperscript{38}.

DLI from the original stem-cell donor can be applied as a treatment for relapse of leukemia after allogeneic HSCT\textsuperscript{8}. DLI can induce remission in up to 80\% of relapsed CML patients. DLI also has beneficial effects in relapsed low grade lymphoma or chronic lymphocytic leukemia (CLL)\textsuperscript{67}, whereas relapsed ALL, AML and myelodysplastic syndrome (MDS) hardly responded and showed an overall survival of less than 20\%\textsuperscript{7,9,14}. The prize for the beneficial effect of DLI therapy is an increased risk to develop acute GVHD, due to the infusion of large numbers of unselected host-reactive donor T cells\textsuperscript{7,9}. The association of the GVL effect with GVHD after DLI further indicates a role of minor H antigens in both the GVL effect and GVHD\textsuperscript{7,8,53}. 
3. Major and minor Histocompatibility antigens

3-1. Major Histocompatibility antigens

The human major Histocompatibility complex (MHC) is designated as HLA in humans. The genes encoding HLA are located on the short arm of chromosome 6. The genes in this region encode cell-surface antigen-presenting proteins and a number of other immune-related proteins\(^68-70\). The HLA antigens are essential elements in immune processes; patients who fail to express HLA are severely immunocompromised\(^71-75\).

HLA proteins can be divided into two groups: the HLA class-I group and the HLA class-II group. This distinction is based upon their molecular structure and immune function. HLA class-I molecules consist of a single transmembrane polymorphic α-chain that is non-covalently associated with a non-polymorphic β2-microglobuline. The HLA class-I (HLA-A, -B, -C) and class-II (HLA-DP, -DQ, -DR) antigens are co-dominantly expressed and differ in their tissue distribution and characteristics in peptide presentation to T cells\(^76,77\). HLA class-I molecules function as antigen-presenting molecules on most nucleated cells. They are assembled in the endoplasmic reticulum (ER), where they are loaded with peptides of about 8~10 amino acids in length. These peptides are generally derived from endogenously synthesized proteins that are broken down by proteasomes. Once digested, these peptides are transported into the ER, where they can bind to class-I molecules. The HLA class-I/peptide complexes are subsequently transported to the cell surface, where they can be recognized by CD8\(^+\) cytotoxic T lymphocytes (CTL)\(^77-80\).

HLA class-II molecules contain two transmembrane chains (one α- and one β-chain), which can both be polymorphic\(^81\). They are expressed on professional APCs as dendritic cells (DC), B cells, activated T cells. HLA class-II molecules are assembled in the ER and transported into endosomal compartments, where the peptides are loaded. These peptides are generally derived from extracellular or membrane-bound proteins that have been internalized by endocytosis/phagocytosis. After endocytosis/phagocytosis, these proteins are degraded in an endocytic compartment, leading to peptides of about 10~30 amino acids in length that can bind HLA class-II molecules. The HLA class-II/peptide complexes expressed on the cell membrane are usually recognized by CD4\(^+\) T helper (Th) cells. These Th cells subsequently stimulate either antibody-producing B cells\(^80,82\) or activation/expansion of CTLs.

Immune responses against incompatible HLA in HSCT is associated with severe post-
transplant complications such as GVHD; the frequency of acute GVHD is directly related to the degree of mismatch between HLA-proteins\textsuperscript{39,50,58,83,84}. Therefore, an important factor to improve HSCT outcome is related with the accuracy of histocompatibility testing and HLA matching, in particularly when transplanting between unrelated individuals.

3-2. Minor Histocompatibility antigens

Following HLA-matched HSCT, donor T-cell immune responses are generally directed against disparate peptides presented by HLA molecules. These peptides arise from polymorphic self proteins, i.e. they can differ between the IRD and the recipient. These polymorphic immunogenic peptides are designated as minor H antigens. Human minor H antigens are encoded on the autosomal chromosomes or on the Y-chromosome (see table 1). In most cases, the polymorphism is the result of a single nucleotide polymorphism (SNP)\textsuperscript{85}. The human genome contains a large number of these SNPs. Only non-synonymous SNPs results in polymorphic proteins and peptides due to a different amino acid after translation of the mRNA. These (single) amino acid differences may influence the intracellular processing of the peptide\textsuperscript{86}, affect TAP transport efficiency\textsuperscript{87}, alter TCR recognition\textsuperscript{88}, or change the binding affinity to certain HLA molecules\textsuperscript{89}. Also, peptide expression can vary due to deletion of the gene encoding the source protein\textsuperscript{90}.

Depending on the HLA-binding properties, minor H peptides are expressed on the cell surface by HLA class-I or class-II molecules and are recognized by HLA-restricted alloimmune donor T cells\textsuperscript{80,81}. Thus, minor H antigen presentation is HLA-allele-restricted. The currently identified minor H antigens and their characteristics are listed in table 1.

Minor H antigen expression can be ubiquitous or limited to specific tissues and cells, as will be outlined in detail below. The epitopes recognized in a GVL response may involve both broadly expressed host alloantigens, which can induce GVHD and GVL, and tissue and cell restricted minor H antigens, which induce only GVL. In general, most of the Y-chromosome encoded minor H antigens, the HY antigens, are broadly expressed. Clinical reports demonstrate that HY antigens contribute to both GVHD\textsuperscript{91,92} and to GVL activity\textsuperscript{91}. The involvement of broadly expressed HY antigens in GVHD has been confirmed by in vitro experiments, i.e. CTLs directed to broadly expressed minor H antigens lyse, amongst others, cell types affected during GVHD, such as fibroblasts, melanocytes, and keratinocytes\textsuperscript{93}. 
Table 1. The currently molecularly identified minor H antigens encoded on the Y-chromosome genes and on the autosomal genes.

<table>
<thead>
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<th>minor H antigen</th>
<th>HLA restriction</th>
<th>Gene</th>
<th>Peptide</th>
<th>Chromo -some</th>
<th>Tissue distribution</th>
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Moreover, HY-specific T cells can be detected in male recipient of a female HSCT. CTLs specific for the broadly expressed HY antigen can also be detected in association with leukemia remission.

GVL activity is believed to be mainly associated with hematopoietic system-specific minor H antigens. A number of autosomally encoded minor H antigens, as HA-1 and HA-2, are exclusively expressed on the hematopoietic cells, including leukemic cells and leukemic progenitor cells. CTLs specific for the hematopoietic system-restricted minor H antigens HA-1 and HA-2 are therefore capable of lysing leukemic cells. Clinically, these antigens coincide with remission of hematological malignancies after DLI. Strikingly, some of the hematopoietic system-restricted minor H antigens are also expressed on a variety of solid tumor cells, indicating that these antigens are relevant for the graft-versus-tumor (GVT) activity after HSCT for solid tumors.

4. Minor Histocompatibility antigen-specific T cells in graft-versus-host disease

4-1. The role of minor Histocompatibility antigen-specific T cells in graft-versus-host disease

As outlined above, incompatibility for minor H antigens may play an important role in the development of GVHD. Indeed, clinical studies demonstrated that GVHD following IRD HSCT significantly correlates with the disparity for a single minor H antigen mismatch, i.e. HA-1, 2, 3, 4, 5, or HY. Thus, the occurrence of GVHD after IRD HSCT seems to be directly correlated with minor H-antigen disparity. However, the diversity of minor H antigen-specific T-cell responses and the contribution of each separate response to the donor-derived immune responses are unclear. So far, the donor-derived immune response seems to be directed against a limited number of immunodominant minor H antigens.

The tissue expression patterns of minor H antigens have been studied extensively, using minor H antigen-specific CD8+ CTLs and CD4+ Th cells. These cells were isolated from the peripheral blood of patients with severe GVHD. Using minor H antigen-specific CTLs, the cell membrane expression of a number of minor H antigens was analyzed. Some minor H antigens, such as HY, HA-3, HA-8, and UGT2B17, appeared to be expressed on all tissues, and thus these antigens are
likely targets for GVHD. Indeed, clinical results show that male patients receiving HSCs from a female donor have a higher risk of developing GVHD. High frequencies of circulating male-associated HY-specific T cells were detected in the peripheral blood of male patients during acute GVHD using tetrameric HLA/minor H antigens peptide complexes. In addition, studies in an in situ ex vivo skin explant model demonstrated that the HY-specific T cells could infiltrate male skin and induce GVH-like reactions. Thus, ubiquitous minor H antigens such as HY are likely to be causatively involved in GVHD.

The other minor H antigens, such as HA-1, HB-1, ACC-1, ACC-2, SP110, PANE1, and LRH-1 show a restricted cell-membrane expression. They are expressed on hematopoietic cells including leukemic cells, but not on tissues and cells of non-hematopoietic origin. Therefore, these minor H antigens can potentially enhance the GVL effect with low GVHD. In particular for minor H antigen HA-1, extensive cellular functional analyses and mRNA studies showed its exclusive expression on cells of the hematopoietic system and on solid tumors. Thus, based upon this restricted tissue distribution, HA-1-specific CTLs are considered to attack the malignant and hematopoietic cells while leaving the non-malignant cells intact. Indeed, in vitro studies confirmed the correlation between hematopoietic-restricted minor H antigen-specific CTLs and GVL but not GVHD. However, some clinical studies demonstrated that mismatching for minor H antigen HA-1 might still be associated with GVHD in adult recipients of HLA-identical sibling transplantation. As the HA-1 antigen is not directly expressed on the GVHD target tissues, the GVHD induction by HA-1-specific CTLs may be dependent on the presence of remaining patient’s hematopoietic cells in the GVHD target organs. This latter requirement also implies that the mechanisms of GVHD induction by hematopoietic versus ubiquitous minor H antigens may show essential differences, i.e. donor T cells directed at ubiquitously expressed minor H antigens may mediate the tissue damage by direct killing of the minor H antigens presenting cells, whereas activation of T cells specific for hematopoietic minor H antigens by recipient’s APCs in the tissues may result from production of inflammatory cytokines, epitope spreading or collateral damage.
4-2. The interaction between antigen presenting cell and minor Histocompatibility antigen-specific T cells in graft-versus-host disease

APCs play an important role in the development of GVHD\textsuperscript{144,145}. Some studies demonstrated that the inactivation of APCs in mouse BM chimeras prevented the induction of GVHD after HLA-matched, minor H antigens-mismatched transplantation, indicating a requirement for these host APCs in the GVHD pathogenesis\textsuperscript{144,146}. Although donor APCs exacerbate GVHD in some animal models, they seem to be less important than host APCs, as cross presentation of host antigens by donor-derived APCs is not required for the induction of severe GVHD\textsuperscript{147}. Host APCs may promote the induction of GVHD, whereas donor APCs may contribute to the perpetuation of tissue injury in chronic GVHD\textsuperscript{148,149}. A possible explanation could be that host APCs present minor H antigens more efficiently than donor APCs, since host APCs process and present the endogenous host minor H antigens without the necessity for antigen uptake from an exogenous source\textsuperscript{150}.

The lifespan of host APCs after allogeneic HSCT is limited; host APCs are generally replaced by donor APCs and gradually disappear from the host following allogeneic HSCT\textsuperscript{151}. In human, host Langerhans cells (LCs) will generally disappear from skin approximately 14~21 days after allogeneic HSCT\textsuperscript{151}, but may persist longer depending on the conditioning regimen, with up to 40 days after full-intensity and 100 days after reduced-intensity\textsuperscript{151}. Thus, during that period, host LCs are available to prime donor T cells for minor H antigen-specific responses. In general, host LCs completely disappear after 1 year\textsuperscript{151}. As a result of the disappearance of host APCs at the various sites of the patient’s body, the expression of the patients’ hematopoietic-restricted minor H antigens also declines, because the hematopoietic system is of donor type after HSCT.

5. Effector mechanisms of graft-versus-host disease

GVHD target organ damage is associated with influx of lymphocytic effector cells, such as CD8\textsuperscript{+} CTLs, CD4\textsuperscript{+} T cells, NK cells, and with an increased local expression inflammatory molecules, including tumor necrosis factor-\textalpha (TNF-\textalpha), interferon-\gamma (IFN-\gamma) and reactive oxygen species\textsuperscript{152}. Cellular effectors require cell-cell contact to kill the target cells during GVHD by activation of the perforin/granzyme\textsuperscript{153,154}, Fas/FasL (Fas ligand)\textsuperscript{154} or TNFR/TRAIL (TNF-related apoptosis-inducing ligand) pathways\textsuperscript{155}. In general the Fas/FasL
and the perforin pathways preferentially utilize CD4\(^+\) T cells and CD8\(^+\) T cells, respectively\(^{153,156-158}\). Both pathways may contribute to GVHD pathophysiology\(^{158-160}\).

5-1. Cytotoxic effector mechanisms in graft-versus-host disease

Following HSCT, donor effector T cells get activated and expand in the draining lymph nodes. Subsequently, these alloreactive T cells migrate to the GVHD target organs, where they can damage the target tissues of GVHD. This tissue destruction can be mediated through both direct cytotoxic activity and the recruitment of other leukocytes\(^5\). Intervening in these effector pathways may be a useful strategy to prevent or reduce GVHD severity\(^5\).

Several mouse models demonstrate the role of the Fas/FasL and perforin/granzymes pathways in the development of GVHD, using mice that are deficient for FasL (gld mice), perforin, or granzyme B as donors, or by the in vivo administration of neutralizing anti-FasL antibodies\(^{132,159,161,162}\). Similarly, adoptive transfer of T cells from perforin-deficient mice into MHC class I-, class II- or minor H antigen-mismatched mice, improves survival when compared to transfer of wild-type T cells\(^{132,159,163,164}\). This prolonged survival indicates that perforin- and FasL-deficient mice possess a severely impaired capacity to induce GVHD and demonstrates the major roles of Fas and perforin pathways in GVHD\(^{155}\). These observations are supported by human studies in which elevated levels of soluble Fas were found in the serum of GVHD patients\(^{165-168}\) and studies in which perforin and granzyme B could be observed in GVHD lesions\(^{169}\) and in the supernatants of 96 hours pretransplant mixed lymphocyte cultures (MLC)\(^{170}\). In a mouse study, GVHD in MHC class-I and class-II disparate mice appeared to be mediated only by those CD4\(^+\) and CD8\(^+\) cells that utilized Fas/FasL pathway\(^{154}\). Moreover, Schmaltz et al. suggested that the perforin pathway was preferentially used by CD8\(^+\) T cells to mediate GVL\(^{171}\). Additionally, Fas/FasL- and granzyme-independent mechanisms can induce GVHD, as demonstrated in studies where administration of high numbers of T cells from FasL and perforin knockout mice into MHC- or minor H antigen-mismatched strains induce severe GVHD\(^{172}\). In these cases, the cytokines may be involved in inducing GVHD.

5-2. Cytokines in graft-versus-host disease pathophysiology

Inflammatory cytokines can enhance the effect of cytolytic effector cells in GVHD. These
cytokines not only synergistically amplify local tissue injury by attracting immune cells, but may also directly damage GVHD target tissues\textsuperscript{173}. Effector CD8\textsuperscript{+} T cells secrete IFN-\textgreek{\textgamma} and TNF-\textalpha. These cytokines can kill tumor cells and recruit additional effector cells. CD4\textsuperscript{+} T cells can differentiate into Th1 and Th2 type T cells. Th1 cells produce IL-2, IFN-\textgreek{\textgamma}, and TNF-\textalpha, which are involved with cell-mediated cytotoxicity, and Th2 cells secrete IL-4, IL-5, IL-10, and IL-13, which are associated with antibody-production by regulating B cell proliferation. Although Th1 cytokines generally induce GVHD efficiently, the balance of Th1 and Th2 cytokines is important in the immunopathogenesis of GVHD, but remains incompletely understood\textsuperscript{174}.

Clinical and experimental data support a role for cytokines in GVHD. A so-called “cytokine storm” is considered to underlie the initiation of acute GVHD\textsuperscript{175}. The cytokine storm starts with tissue damage caused by the conditioning regimen. Subsequently, lipopolysaccharide (LPS) and other microbial products are released from the intestines and other host tissues, leading to secretion of IFN-\textgreek{\textgamma}, TNF-\textalpha, IL-1 and nitric oxides\textsuperscript{176-178}. The proinflammatory cytokines IFN-\textgreek{\textgamma} and TNF-\textalpha have been associated with acute GVHD\textsuperscript{179,180}. The role of IFN-\textgreek{\textgamma} in GVHD is, however, controversial\textsuperscript{181}; exogenous administration of IFN-\textgreek{\textgamma} or T cells from IFN-\textgreek{\textgamma}-deficient donors results in a reduction and enhancement of GVHD, respectively\textsuperscript{182,183}, whereas high levels of IFN-\textgreek{\textgamma} production by both CD4\textsuperscript{+} and CD8\textsuperscript{+} donor effector T cells early after BMT can limit the severity of acute GVHD in recipient mice after myeloablative conditioning\textsuperscript{184}. Clinically, recipients homozygous for an IFN-\textgreek{\textgamma} expression variant with low production after \textit{in vitro} stimulation were more likely to develop severe acute GVHD\textsuperscript{185}. Following HLA-identical HSCT, the soluble \textalpha chain of the IL-2 receptor (sIL-2R\textalpha) levels were increased during chronic GVHD\textsuperscript{186}. There is experimental and clinical evidence for the involvement of TNF-\textalpha in GVHD. Serum TNF-\textalpha levels are elevated during acute GVHD\textsuperscript{187}. Treatment of mice with TNF-\textalpha antibodies can inhibit GVHD\textsuperscript{187}. Moreover, clinical trials treating GVHD patients with TNF-\textalpha antibodies limit the GVHD symptoms\textsuperscript{160,188-190}. As TNF-\textalpha is able to activate the host APCs\textsuperscript{191}, these activated APCs display an increased expression of MHC and costimulatory molecules on their cell surface, leading to an enhanced antigen presentation to donor T cells\textsuperscript{191,192}. This enhanced antigen presentation may promote the GVHD pathogenesis.

In humans, the proinflammatory effect of the above-described cytokines may be dampened by
anti-inflammatory cytokines. Anti-inflammatory candidates are IL-10, IL-1 receptor antagonist (IL-1ra), and TGF-β. The serum levels of IL-10 and of the cytokine-related IL-1ra were elevated during both infections and chronic GVHD\textsuperscript{186}. Moreover, recipients with a genetic variant of IL-10 leading to an increased expression, have a lower risk to develop GVHD\textsuperscript{43,185}. Experimental data have demonstrated that transforming growth factor β (TGF-β) plays a regulatory role in the immune system\textsuperscript{193}, attenuates acute GVHD\textsuperscript{186}, but may lead to chronic GVHD\textsuperscript{194}. Thus multiple cytokines are important in GVHD pathogenesis and regulation.

6. Scope of this thesis
The studies in this thesis explore the complex mechanisms of human GVHD induced by ubiquitous and hematopoietic restricted minor H antigens using an \textit{in situ} skin explant assay and using skin samples obtained from GVHD patients after HLA-matched minor H antigen-mismatched SCT.

In chapter 2, we describe a new methodology that allows staining with tetrameric MHC/peptide complexes combined with \textit{in situ} intracellular multiple color antigen staining using Confocal Laser Scanning Microscopy (CLSM) technology on cryosections. We first optimized the MHC/peptide tetramer CLSM technique for intracellular antigens using the cryopreserved male skin explants incubated with HY-specific CTLs. Using the newly developed methodology, we demonstrated antigen-specific up-regulation of proliferation marker Ki67 in combination with clustering of Granzyme B in minor H antigen-specific T cells upon \textit{in situ} recognition of their specific target cells.

In chapter 3, we extended the staining methodology and analyzed GVHD skin samples obtained from pediatric patients who received minor H antigen HY-mismatched SCT. Clinical skin samples obtained from sex-mismatched HSCTs were stained with specific MHC/peptide dextramers to, for the first time, visualize minor H antigen-specific T cells \textit{in situ}. Tricolor MHC-dextramer-CLSM technologies were used to further analyse the phenotype of minor H antigen-specific T cells and to identify their target cells.

In chapter 4, we investigated the role of HA-1-specific CTLs in the development of GVHD and whether recognition of infiltrating APCs by HA-1-specific CTL results in GVH reactions (GVHR). To determine the \textit{in situ} activities of CTLs specific for hematopoietic minor H
antigens, we modified the skin explant assays, enabling APCs to infiltrate into the skin sections. The skin explants prepared without CTLs were analyzed by immunohistochemistry to determine semi-quantitatively the degree of skin infiltration by the APCs. Furthermore, we investigated whether the grade of the histopathological damage induced by minor H antigen-specific CTLs can be correlated with the degree of hematopoietic-cell infiltration, in order to estimate the contribution of hematopoietic APCs to the GVHR.

In chapter 5, we investigated the effect of minor H antigen mismatching on the clinical outcome of HLA-matched and HLA-identical SCT in a multi-center study. Analyses were performed per minor H antigen and in groups based upon biological characteristics as tissue distribution and HLA restriction. We investigated whether mismatching for broadly expressed minor H antigens or for hematopoietic minor H antigens influence GVHD incidence or relapse rates. Moreover, we investigated whether the effect of minor H antigen mismatching on relapse depended on the development of GVHD.

In chapter 6, the overall results are summarized and discussed.
CHAPTER 1

Reference list

61. Nestel, F.P., Price, K.S., Seemayer, T.A. & Lapp, W.S. Macrophage priming and lipopolysaccharide-
CHAPTER 1


