The handle http://hdl.handle.net/1887/20472 holds various files of this Leiden University dissertation.

**Author:** Kim, Yeung-Hyen  
**Title:** Mechanistic studies on human minor histocompatibility antigens in graft-versus-host disease  
**Issue Date:** 2013-01-30
Chapter 6

Summary and General discussion
Contents

1. Development of methods to detect of antigen-specific T cells in situ

2. The involvement of minor Histocompatibility antigen-specific T cells in skin GVHD
   2-1. The ubiquitously expressed minor Histocompatibility antigen HY in skin GVHD
   2-2. The hematopoietic-restricted minor Histocompatibility antigen HA-1 in skin GVHD

3. Minor Histocompatibility antigen mismatching in GVHD and GVL

4. Summary of the relevant results
1. Development of methods to detect of antigen-specific T cells in situ

MHC class-I/peptide tetramers allow direct visualization and quantification of antigen-specific cytotoxic T cells\(^1\). Since the first report on the generation of tetrameric MHC class-I/peptide complexes, the number of basic and clinically orientated tetramer-based studies has grown exponentially\(^2\). Nowadays, tetramer staining in combination with staining for other molecules such as cytokines, chemokines, chemokine receptors, and costimulatory molecules, can be performed to evaluate functional aspects of antigen-specific T cells and their potential role in curative and pathogenic immunological processes. However, these analyses on peripheral T cells yield no information on the anatomical localization. The question whether these T cells are indeed infiltrating the target tissues or rather are only present in the peripheral blood and lymphoid tissues, have been studied and discussed in this thesis.

Various methods can be applied for the detection of antigen-specific T cells in both experimental samples and in patient’s specimens. One efficient method uses MHC/peptide tetramer technology that allows enumeration, characterization, and isolation of antigen-specific T lymphocytes in and from normal or pathologically affected tissues\(^1,3,4\). MHC/peptide tetramers were used in both mouse and human models to visualize \textit{ex vivo} virus-, tumor-, and minor H antigen-specific T cells\(^5,6\). In the latter studies, antigen-specific T cells were visualized in suspensions isolated from peripheral blood mononuclear cells PBMC. Using confocal laser scanning microscopy (CLSM), mouse virus- and tumor-specific T cells can be visualized \textit{in situ} upon MHC tetramer staining in viable and unfixed tissues sections\(^7,8\). However, the \textit{in situ} application of tetrameric MHC/peptide complexes in viable tissue sections has several shortcomings, i.e. 1) co-staining of the tetramer positive cells for various intracellular molecules is impossible, 2) multiple analyses on one single biopsy cannot be executed, 3) the biopsies cannot be stored before analyses. A different but more laborious method allows MHC tetramer staining in paraformaldehyde fixation samples, but this method requires the presence of clustered antigen-specific T cells\(^9\).

In \textbf{chapter 2}, we successfully developed and validated a new \textit{in situ} staining methodology. This new methodology facilitates investigations on the anatomical localization of T cells with a defined specificity by using tetrameric MHC/peptide complexes. Moreover, it permits staining of intracellular and intranuclear markers in cryopreserved human samples that are pre-stained with tetramers. This methodology solves the above-mentioned problems regarding
co-staining and multiple analyses on a single biopsy. Based on this methodology, we showed that most HY-specific CTLs that were invading in the ex vivo in situ skin explant assay, were positive for the intranuclear activation marker Ki67, suggesting their active proliferation. No activation of HY-specific CTL was observed in skin explant assays with skin tissues of female volunteers. In addition, we also demonstrated that cytoplasmic Granzyme B signals co-localized in part with TCR in polarized way. The latter observation matches with previous findings, i.e. CTL will polarize their TCR and Granzyme B at the interface with their specific target cells, where they excrete cytotoxic granules\textsuperscript{10}.

Apart from Granzyme B there may be other cell-death inducing pathways, including FAS-mediated apoptosis. Such pathways could in principle be analyzed in combination with the here-described technique; CTL-induced caspase activation in target cells can be detected via specific cleavage of fluorogenic caspase substrates\textsuperscript{11}. Thus, this technology can be combined with tetramer staining to detect apoptosis induced by minor H antigen-specific CTLs.

Our tetramer methods failed, however, to stain human materials after cryopreservation, in contrast to a mouse study on cryopreserved tissues\textsuperscript{12}. The main reasons for this discrepancy may be 1) differences in the strength and specificity of the antigenic stimulation, 2) differences in target tissues, and 3) differences in the origin of the samples, i.e. mouse versus human. Moreover, in these mouse studies, modifications of the epitope were essential to successfully detect these antigen-specific T cells, indication the need for a high avidity TCR/tetramer interactions. Thus, freezing biopsies before tetramer staining may prohibit the detection of single antigen-specific T cells.

Nowadays, antigen-specific T lymphocytes can also be detected by direct staining of human tissue cryosections using multimeric dextran-coupled peptide/MHC complexes in combination with a modified peptide antigen\textsuperscript{13,14}. These modifications of the epitope may potentially enhance both the affinity and avidity to the TCR.

In chapter 3, we developed methods to detect of HY-specific T cells in skin biopsies by direct staining on cryopreserved tissues using dextrameric reagents loaded with the unmodified A2/HY peptide. This methodology allows ex vivo in situ staining of previously frozen human tissue and thus facilitates analysis of a single inflamed tissue biopsy with multiple dextrameric MHC/peptide complexes. We first validated and applied this methodology to assess the presence of minor H antigen HY-specific T cells in the skin of
pediatric GVHD patients after gender-mismatched HSCT. Indeed, the dextrameric HLA-A2/HY peptide complexes allowed repeated staining and analyses of infiltrating T cells in cryopreserved tissues. These results thus enable, for the first time, the performance of retrospective analyses of stored clinical GVHD samples.

2. The involvement of minor Histocompatibility antigen-specific T cells in skin GVHD

2-1. The ubiquitously expressed minor Histocompatibility antigen HY in skin GVHD

In the HLA-identical HSCT setting, the development of GVHD is considered to be caused by donor T cells specific for ubiquitously expressed minor H antigens such as the Y-chromosome-derived HY minor H antigens. While numerous clinical results show that gender mismatching significantly affects HSCT outcome, the in situ presence of HY-specific T cells in GVHD-affected tissues had thus far not been demonstrated. To analyze the presence of HY-specific T cells in GVHD-affected tissues, the frozen patient skin materials were stained with HLA-A2/HY-dextramers and analyzed by CLSM. The percentage and absolute number of HLA-A2/HY-dextramer+ CD8+ T cells were significantly higher in male recipients of a female HSCT. However, we failed to detect circulating HY-specific T cells in patient PBMC collected in parallel with the skin biopsies. Earlier, the presence of HY-specific T cells was reported in the peripheral blood of adult male HSCT patients with acute GVHD after non-T-cell depleted BM graft from female donors. These contradictory results are possibly due to significant differences in the applied HSCT protocols, such as the type and composition of the applied stem-cell grafts and the pre- and post-HSCT applied immune suppression regimens. Moreover, the amount of PBMCs required for a proper evaluation and the low level of immune reconstitution in these pediatric patients at the time of GVHD were limiting factors. Nonetheless, our study in chapter 3, points out that significant numbers of HY-specific T cells home to the skin at an early stage of acute GVHD, even at time points when the peripheral immune reconstitution is low. Nonetheless, our results strongly argues for the first time for the active involvement of HY-specific T cells in GVHD affected male skin by female derived HY-specific T cells.

The above-described observation of HY-specific T cells present in GVHD skin but not in peripheral blood, raises the question whether these relatively high number of HY-specific T
cell in the GVHD skin result from local expansion or from specific mechanism for infiltration. Skin-infiltrating CD4$^+$ and CD8$^+$ T cells did not express the proliferation marker Ki67$^1$. Likewise, the HLA-A2/HY-dextramer$^+$ CD8$^+$ T cells described in chapter 3 did not express the proliferation marker Ki67.

The presence of skin-homing CD4$^+$ and CD8$^+$ T cells was previously described in an independent cohort of pediatric patients developing GVHD after infusion of an allogeneic HSCT$^1$. It is known that CD4$^+$ T cells and, to a lesser extent, also the CD8$^+$ T cells, can express CCR10. This chemokine receptor is involved in cellular trafficking specifically to the skin. It guides T cells towards the dermal-epidermal junction via local secretion of chemokines such as the CCR10 ligand CCL27 (CTACK)$^1$. CCL27 can be produced by keratinocytes, particularly after exposure to TNF-α and CCL17 (TARC)$^1$. Collectively, our observations suggest that the high numbers of HY-specific T cells present in acute GVHD-affected skin, may result from preferential migration than from local proliferation.

To further characterize the infiltrating HY-specific T cells, skin sections were stained with granzyme B. The highly granzyme B$^+$ cells residing at the basal layer of the epidermis, expressed CD8 at very low densities. Speculatively, these cells may represent locally activated CD8$^+$ T cells that seem to have almost completely internalized their CD8 coreceptor upon antigen recognition. In general, TCR stimulation-induced cell-cycle progression is required for the induction of cytotoxic granules in both naïve and antigen-experienced CD8$^+$ T cells$^2$. Consequently, granzyme B$^+$ CD8$^+$ T cells residing further away from the dermal-epidermal junction, may not yet have been activated appropriately.

The influx of HLA-A2/HY-specific T cells into the skin may have been facilitated by helper T cells. These helper T cells could have arisen due to additional major HLA or minor H antigen mismatches. As all pediatric patients and their donors in this study were HLA typed, we analyzed the HLA mismatches between the pairs. All recipient/donor combinations displayed at least one HLA-DPB1 mismatch in the GVH direction. Although still a subject of debate, a recent study performed in the MUD HSCT setting reported an association between HLA-DPB1 mismatching and the risk to develop acute GVHD$^3$. These observations are supported by reports demonstrating that HLA-DP-restricted CD4$^+$ T cells can be cultured from acute GVHD affected skin biopsies derived from HLA-DP-mismatched HSCT recipients$^4,5$. Therefore, we speculate that skin-infiltrating CD4$^+$ T cells specific for the
mismatched HLA-DP antigens facilitate the influx and local activation of T cells specific for broadly expressed minor H antigens, such as HY, in minor H antigen-mismatched HSCT settings. Indeed, a recent retrospective study showed a synergistic effect of HY mismatching and HLA-DP mismatching in relation to the development of severe acute GVHD (Spierings et al. manuscript in preparation).

GVHD target organ infiltrating CD8+ T cells may be triggered by three types of dermis-residing professional APCs; 1) CD14+ CD1a- dermal DCs, 2) CD14+ CD1a+ dermal DCs or 3) resident macrophages. These skin-residing DCs of host-origin are generally completely replaced by cells of donor origin at 40 days after HSCT. In contrast, residual dermal macrophages are replaced at a significantly slower rate. If correct, these three types of host APC would still be present in the skin of biopsied patients analyzed in chapter 3. Haniffa and colleagues reported that skin-infiltrating CD3+ T cells interact with perivascular macrophages. Furthermore, these authors provided evidence that dermal DCs and macrophages are both able to induce proliferation, cytokine-release, and expression of activation markers by allogeneic CD8+ T cells. However, in chapter 3, we could not detect any interaction between dermal CD1a+ DCs and HLA-A2/HY-specific CD8+ T cells in male pediatric skin tissue who received a female HSCT, suggesting that host-derived DCs in the skin do not play major role in the local expansion or infiltration of these T cells. Alternatively, the relevant DCs disappear upon ex vivo tissue preparation.

The early appearance of HY-specific T cells in the skin questions whether these, presumably female donor-derived, T cells arise from naïve or memory T cells that are co-transferred with the hematopoietic stem cells. In approximately 50% of all healthy parous blood donors tested after delivery of male offspring, HY-specific T cells have been reported. These pregnancy-induced T cells remain detectable in peripheral blood samples collected many years after the last delivery. It is conceivable that such T cells also end up in hematopoietic stem-cell products. If this assumption is correct, this phenomenon could explain why male recipients of female stem cells have an increased risk to develop acute GVHD, as reviewed. The availability of reliable tools to study the presence of HY-specific CD8+ T cells in both pre- and post-HSCT collected peripheral blood as well as in tissue specimens of male recipients of female stem cell products have now opened possibilities to address the issue
whether pregnancy-induced female graft-contaminating HY-specific T cells indeed contribute to acute GVHD in male recipients after gender mismatched allogeneic HSCT.

2-2. The hematopoietic-restricted minor Histocompatibility antigen HA-1 in skin GVHD

The effect of hematopoietic system-specific minor H antigen mismatching on GVHD has been studied in great detail for minor H antigen HA-1. The results vary per study; some studies report an association between HA-1 mismatching and the development of GVHD whereas others do not\textsuperscript{27-30}.

In chapter 4, we explored the reported association of HA-1 mismatching using the \textit{ex vivo in situ} skin explant model. Our hypothesis to explain the development of acute GVHD for patients with an HA-1 mismatch, is the expression of the HA-1 antigen on the host APCs, i.e. Kupffer cells in the liver, Langerhans (LH) cells in the skin, or small bowel macrophages\textsuperscript{29}. Indeed, human skin has been shown to harbor various DC subpopulations, both in steady state and in inflammation\textsuperscript{31}. However, our earlier study showed that HA-1-specific T cells neither infiltrated HA-1-positive skin explants nor induced GVHR-related tissue damage in the \textit{ex vivo in situ} skin explants model\textsuperscript{32}. We confirmed these results in chapter 4; the restricted expression of HA-1 on hematopoietic cells and the absence of minor H antigen-expressing APCs in skin tissue are the explanations for the latter results. Analyses of the skin explant samples revealed that our skin explant samples were devoid of endogenous dermal DCs. The absence of DCs in our skin explant model may be explained by the fact that our skin explants model includes culturing steps for 96 hours. Our data show that these relatively long culturing time span allows the migration of the endogenous dermal DCs out of the skin (see Chapter 4 Supplementary Figure S2).

Given the above, reconstitution of DCs in the skin explant model was crucial in this study. There are several ways to \textit{in vitro} differentiate and mature monocyte-derived DCs. Depending on the method used, the generated DCs vary substantially in their phenotypic and functional properties, e.g. chemotraction of T cells. In our model, we chose the IL-4/GM-CSF differentiated mature DCs type for various reasons. First, DCs that migrate to the dermis and not the epidermis were preferred to create a model system that closely matches the \textit{in vivo} skin GVHD situation, where skin GVHD is observed as a phenomenon occurring in the dermis. As LH cells are located in epidermis, this type of DCs would lead to an irrelevant
GVHD model system and to incorrect interpretation of the data. Our results show that the *in vitro* differentiated DCs indeed migrate to the dermis. These DCs are therefore preferred above LH-like DCs. Second, the antigen presenting capacity of LH cells is a matter of debate\(^3\). The high antigen-presenting capacity is an established characteristic of mature IL-4/GM-CSF differentiated DCs. Third, we could detect CFDA-SE-positive cells in the skin biopsies incubated with mature DCs but not in those incubated with immature DCs. These mature DCs share phenotypic characteristics resembling the phenotype of dermal CD1a\(^+\) DCs; a high expression of CD1a, CD86, and HLA-DR, and absent of CD14.

Chemoattractive signals provided by the DCs, are important for the influx of T cells. Thus, it would be preferable to analyze chemokine expression patterns *in situ* on skin of patients with skin GVHD, together with the expression of chemokine receptors on skin-infiltration T cells *in vivo*. The role of chemokines and chemokine receptors in the infiltration process has been discussed in section 2-1 in detail. Our current data, however, point out that, apart from chemoattractive signals, a cognate interaction between autologous residual HA-1-expressing dermal DCs and skin cells, may be crucial for infiltration, as absence of either the immunogenic HA-1\(^{11}\) allele or the HLA-A2 molecule prohibits infiltration and activation. In some cases, we occasionally observed GVH reactivity in skin of HA-1\(^+\), but never in HA-1\(^-\) individuals, suggesting that non-hematopoietic cells can acquire HA-1 antigen from hematopoietic sources. These observations match with the fact that HA-1-specific T cells were activated in HLA-A2\(^+\) HA-1\(^-\) skin samples that were reconstituted with HLA-A2\(^-\) HA-1\(^+\) DCs. As HA-1-mediated GVHD could not be attributed to side damage of APC-directed cytolysis in our model, we speculate that direct recognition of HA-1 derived from hematopoietic cells cross-presented by skin cells, is essential to destruct skin tissue during GVHD.

3. **Minor Histocompatibility antigen mismatching in GVHD and GVL**

Some clinical results show that HLA-matched minor H antigen-mismatched transplant recipients have an increased risk for developing GVHD and a poorer survival when compared to minor H antigen matched recipients\(^17\). However, hematopoietic-restricted minor H-antigen mismatches have been reported to exert a beneficial effect, i.e. lower relapse rates due to a GVL effect\(^29,34\). In chapter 5, we therefore investigated the effect of minor H antigen-
mismatching on the occurrence of GVHD and of relapse after MUD or IRD HSCT. We observed an effect of mismatching for HA-8 on GVHD in the IRD HSCT, but not in MUD HSCT. The HA-8 observations are in line with previous reports on IRD transplantation and MUD transplantations, suggesting a differential influence of HA-8 mismatching on GVHD. Apparently, HA-8 disparities are only of clinical relevance under HLA-identical conditions, but not in MUD HSCT, where HLA mismatches are likely to exist. Although statistically not significant, the minor H antigen HLA-A2/HY seems to share these characteristics.

Counter intuitively, matching for HY in HLA-DQ5 showed a trend for an increased risk for GVHD as compared to HY-mismatched HLA-DQ5 pairs. Despite the fact that both the HLA-DQ5/HY epitope and the HLA-DR15/HY epitope are derived from the DDX3Y protein, mismatching for HY in HLA-DR15-positive IRD pairs tends to an increased GVHD incidence. These differential effects are unlikely influenced by cell-biological properties of the mRNA transcripts or the protein, although antigen-processing differences cannot be excluded. Thus, our data reveal that HLA/minor H peptide complexes vary in their immune responses. As yet, we have no explanations why gender matching in HLA-DR15 but not mismatching in the HLA-DQ5 patient/donor pairs increases the risk to development of GVHD. Neither can we rule out that we are looking to an epiphenomenon.

Recently, a relatively small study on HA-1 mismatching and GVL in CML patients, elucidated that the GVL effect of HA-1 mismatching was driven by GVHD. In chapter 5, we investigated the effect of GVHD on GVL, independent of underlying disease and extended to all hematopoietic system-restricted minor H antigens. Most importantly in our underlying analysis, GVHD status is used as a time-dependent risk factor. We observed that mismatching for hematopoietic-restricted minor H antigens per se did not reduce the relapses rates, neither in IRD HSCT nor in MUD HSCT. However, when implementing GVHD status as a time-dependent risk factor, the effect of mismatching for hematopoietic-restricted minor H antigens on relapse incidence, relapse-free survival, and overall survival significantly depended on the development of GVHD. Thus, we not only confirm our previously reported interaction between GVHD development and HA-1 mismatching in relation to the GVL effect, but also showed that it accounts to all hematopoietic minor H antigens, in patients with different
original diseases, and, most importantly, that the association between hematopoietic mismatching and GVHD and GVL is even significant in a multicenter analyses.

4. Summary of the relevant results

In this thesis, we have explored the mechanism of minor H antigen-related GVHD. Hereto, we developed and validated new methods to detect and to phenotype minor H antigen-specific T cells on cryo-preserved human tissues.

Using these methods, we investigated the influence of the ubiquitously expressed minor H antigen HY in skin GVHD. This first report on the in situ detection of skin-infiltrating HY-specific T cells in GVHD affected tissue of pediatric patient’s points to an active role of these T cells in the pathophysiology of acute GVHD after gender mismatched HSCT.

Using our skin explant methodologies, we also aimed at gathering insights into the postulated involvement of hematopoietic-system restricted HA-1 in GVHD. This study was challenged by the contradictory results of clinical studies on the influence of HA-1 with GVHD. We showed that recognition of skin samples reconstituted with mature DCs, facilitated infiltration and local activation of HA-1-specific T cells. Although the infiltrated and activated HA-1-specific T cells did not cause skin tissue destruction in our model, our results provide a first step in understanding the reported association of HA-1 mismatching with clinical GVHD.

Finally, we performed a large multi-center population-based study comprising 849 HSCT pairs, investigating the effect of broadly expressed and hematopoietic restricted minor H antigen-mismatching on GVHD and on relapse incidence after HLA-matched and HLA-identical HSCT. We showed that mismatching for broadly expressed autosomally encoded minor H antigens is associated with increased GVHD in HLA-identical, but not in HLA-matched unrelated HSCT. Disparities between donor and recipient for hematopoietic system-specific autosomally encoded minor H antigens do neither influence the development of GVHD nor had an effect on relapse incidence. Importantly however were the significantly lower relapse rates and disease free survival observed in those patients who suffered from GVHD.
CHAPTER 6

Reference list
