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Chapter 4

HA-1 T cells in skin GVHD
Exogenous addition of minor H antigen HA-1+ dendritic cells to skin tissues ex vivo causes infiltration and activation of HA-1-specific cytotoxic T cells

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ABSTRACT

T cells specific for hematopoietic system restricted minor Histocompatibility (H) antigens target normal and malignant hematopoietic cells. Thus, cellular immune responses against the latter miHAs eradicate the recipient’s hematopoiesis including residual leukemic cells after HLA-matched minor H antigen-mismatched stem-cell transplantation (SCT). However, there are controversial reports on the role of HA-1 in the development of graft-versus-host-disease (GVHD) as well. Here, we address the behavior of HA-1-specific cytotoxic T cells (CTLs) in an ex vivo in situ skin explant model wherein HA-1-expressing dendritic cells (DCs) were added as antigen-presenting cells (APCs). Infiltration and activation of HA-1 CTLs occurred only in those cases where both HLA-A2 and HA-1 were expressed, either by the skin or by the DCs, or by the combination of HLA-A2+ skin and HA-1+ DCs. These results point toward the role of recipient’s HA-1+ DCs in the chimeric patient suffering from GVHD after HA-1-mismatched SCT. Although in our model the infiltrated and activated CTLs did not cause skin tissue destruction, our results provide a first step in understanding the reported association of HA-1 mismatching with clinical GVHD.
INTRODUCTION

Hematopoietic stem-cell transplantation (SCT) is an important treatment modality for patients with leukemia and hematologic disorders. Minor histocompatibility antigen (miHA) disparities between the HLA-matched donor and recipient induce minor H antigen-specific donor T cells causing graft-versus-host reactivity (GVHR) resulting in graft-versus-host disease (GVHD) and graft-versus-tumor (GVT) responses. miHAs are polymorphic peptides presented in the context of HLA class I and II molecules. miHAs display either broad or hematopoietic system restricted expression patterns. As for HA-1, extensive cellular functional analyses and mRNA studies showed its exclusive expression on all cells of the hematopoietic system and on solid tumors. Additionally, in an ex vivo human GVHR skin model, no tissue destruction by HA-1-specific CTLs occurred, whereas CTLs specific for the broadly expressed minor H antigen HY caused GVHR reactivity comparable to clinical skin GVHD grade III-IV. The characteristic of differential tissue and cell expression of miHAs dissects the involvements of minor H antigens in GVHD and in graft-versus-leukemia (GVL). Consequently, the hematopoietic system restricted miHAs, for example, HA-1, can be applied for boosting the GVL effect after HLA-matched HA-1-mismatched SCT. HA-1-based clinical protocols, such as cellular adaptive immunotherapy and vaccinations, are currently being implemented in clinical phase I/II trials. The role of HA-1 in inducing GVL responses without causing GVHD, however, is still controversial; some studies report association between HA-1 mismatching and the development of GVHD, whereas others do not. A plausible explanation for the association of HA-1 with GVHD would be the presence of patient’s residual dermal antigen presenting cells (APCs) in the skin after SCT. These APCs cells reside for various times in patient’s skin. Moreover, depletion of the APCs cells prevents skin GVHD, suggesting a role for these cells in the induction of GVHD. Both the contradictory results on the involvement of HA-1 in the development of GVHD, and the current application of HA-1 for boosting the GVL responses, demand thorough investigation on its putative role in the development of GVHD. Here, we show that the association of HA-1 with the development of clinical GVHD after HLA-matched, HA-1-mismatched SCT may be explained by the presence of recipient’s APCs in the GVHD target.
organs or by crosspresentation of hematopoietic cell-derived HA-1 by nonhematopoietic skin cells.

**METHODS**

**Dendritic cells**
Monocyte-derived dendritic cells (DCs) were generated from healthy individuals by culturing peripheral blood-derived CD14+ monocytes with 250 U/mL IL-4 (Genzyme, Cambridge, MA, USA) and 800 U/mL granulocyte macrophage-colony stimulating factor (GM-CSF) (Novartis Pharma BV, Arnhem, The Netherlands) for 6 days as described previously13. On day 6, the DCs were maturated by culturing on irradiated (75Gy) CD40 ligand-transfected mouse fibroblasts14 at a DC to fibroblast ratio of 2.5:1 overnight as described previously15,16. Subsequently, DCs were harvested and phenotypically analyzed by flow cytometry for cell-surface expression of CD14, CD1a, CD86, and HLA-DR. To monitor infiltration, DCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, 0.5 mM; Molecular Probes Europe BV, Leiden, The Netherlands) for 4 min at 37°C in phosphate-buffered saline (PBS). After washing 3 times with PBS supplemented 1% fetal calf serum (FCS) (Gibco BRL, Breda, The Netherlands), the cells were used in the skin explant assays.

**Cytotoxic T lymphocytes and Epstein Barr Virus-Transformed B lymphoblastoid cell lines**
HLA-A2+ HA-1-specific CD8+ CTL clone 1.7 and HLA-A2/HY-specific CTL clone 21-17 were isolated and expanded from peripheral blood from patients after HLA-matched minor H antigen mismatched SCT, as described before17. Epstein Barr virus transformed B lymphoblastoid cell lines (EBV-BLCL) were generated from peripheral blood mononuclear cells (PBMCs) of healthy blood donors. All transformed cell lines were cultured in complete culture medium consisting of Iscoves modified Dulbecco medium (IMDM) (Biowhittaker, Verviers, Belgium) supplemented with 5% FCS, 100 U/mL penicillin, and 2 mM L-glutamine.

**Tetrameric HLA Class I/Minor H antigen peptide complexes**
Allophycocyanin (APC)-conjugated tetrameric HLA-A*0201 molecules in complex with HA-1 peptide VLHDDDLLEA (HLA-A2/HA-1 tetramer), A2/HY peptide FIDSYICQV, or Flu
peptide GILGFVFTL (HLA-A2/Flu tetramer) were prepared as described\(^1\), with minor modifications\(^2\) and stored at -20°C in 0.5% bovine serum albumin (BSA)/16% glycerol. Tetrameric HLA class I-minor H antigen peptide complexes showed specific and sensitive staining of minor H antigen-specific CTLs both in FACS and confocal laser scanning microscopy (CLSM) analysis in vitro (data not shown).

**Antibodies**

Antibodies used were: FITC-conjugated anti-CD8 (1/20 diluted; Becton and Dickinson, San Jose, CA, USA), FITC-conjugated anti-CD3 (1/20 diluted; DAKO A/S, Glostrup, Denmark), anti-CD3 (1/200, DAKO), mouse anti-CD20 (1/500 diluted; DAKO), biotinylated goat antimouse (1/200 diluted; DAKO), goat anti-APC (5 µg/mL) and rabbit anti-PE (5 µg/mL) (both from Biogenesis, Poole, England, UK), Cy3-conjugated goat anti rabbit (1/400 diluted, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), Alexa 633-conjugated donkey antigoat (5 µg/mL; Molecular Probes Europe BV, Leiden, The Netherlands), anti-Granzyme B (1/50 diluted, PE-conjugated GB11; Sanquin, Amsterdam, The Netherlands), rabbit anti-Ki67 (1/200 diluted; DAKO), and streptavidin-labeled Alexa 568 (12.5 µg/mL; Molecular Probes Europe BV).

**Minor H antigen typing**

Peripheral blood was obtained from HLA-typed healthy volunteers after informed consent. PBMCs were isolated by Ficoll density centrifugation. DNA was isolated using the local standard procedures used for HLA typing. miHAs genotyping was performed using the PCR-SSP technique (Invitrogen Corporation, Carlsbad, CA, USA). The details of the minor H antigen typing methodology have been described before\(^2\).

**Skin samples**

After informed consent, punch biopsies of 4-mm diameter were taken from the upper part of gluteal region of healthy volunteers. The skin was anesthetized with 2% xylocaine with 0.12% adrenaline. The skin biopsies were used directly after preparation. All human material was collected by protocols approved by the local ethical committee of the Leiden University Medical Center (P03-135).
Skin explant assay

**DC infiltration into skin explant tissues**
Punch skin biopsies were dissected into 4 equal sections under sterile conditions. Skin sections were placed in 96-well V bottom microtiter culture plates with dermis orientated upwards and cocultured with $5 \times 10^5$ CFDA-SE-labeled mature DCs or medium alone overnight. All skin donors were unrelated to the DC donors and none of the DC-skin combinations were full HLA matched. Assays were performed in a total volume of 200 µL IMDM supplemented with 10% human serum at 37°C and 5% CO$_2$ for 2, 24, 48, and 72 hours (Figure 1A). After serial incubations at various time points, skin explant tissues were formalin-fixed and paraffin-embedded. Subsequently skin explant tissues were sectioned and stained with either hematoxilin/eosin (H&E) or multicolor fluorescence. Presence or absence of DC infiltration was examined by scoring CFDA-SE labeled DCs per section using CLSM.

**Adapted skin infiltration protocol for optimization of infiltration of DC and HA-1-specific CTLs**
Basically, the skin explant assays were performed as described above. To optimize mature DC infiltration without detrimental effects in skin, variable numbers of mature DCs, ranging from $125 \times 10^3$ to $5 \times 10^5$ per well, were cocultured for 24 hours. GVHR was analyzed at the time points 24, 48, and 96 hours, that is, 0, 24, and 72 hours, respectively, after washing of the cocultures (Figure 1D). Subsequently, the skin explant tissues were analyzed by H&E staining to determine semiquantitatively the degree of skin infiltration by mature DCs. Skin tissues of donors with different HLA- and HA-1-phenotypes (HLA-A2$^+/-$ and/or HA-1$^+/-$) were incubated with DCs of different HLA- and HA-1-phenotypes (HLA-A2$^+/-$ and/or HA-1$^+/-$) for 24 hours. After removal of noninfiltrated mature DCs and extensive washing, the skin explant tissues were cocultured with $1 \times 10^6$ HA-1-specific CTLs for 72 hours. Similarly, HLA-A2$^+$ female (HY$^-$) skin with HLA-A2$^+$ male (HY$^+$) mature DCs were incubated with HLA-A2/HY-specific T cells as a positive control. Subsequently, skin explant tissues were either snap frozen in Tissue-Tek OCT compound (Sakura, Zoeterwoude, The Netherlands) for immunofluorescence analyses or fixed in formalin, sectioned, and stained with H&E for histopathologic grading.
Figure 1. Influx of mature DCs causes skin destruction in skin explant tissues. CFDA-SE-labeled mature DCs were incubated with skin explants tissues as indicated. (A) Schematic time schedule of the DC reconstitution experiment. Mature DCs were added to the skin explant assay at the start of the experiments and cocultured until fixation. (B) GVHR scoring of the samples treated as depicted in A. (*P < .05; **P < .001). (C) H&E staining of the skin explant assays executed as depicted in A. Scale bar represents 10µm. Black arrows indicate dermal-epidermal cleft formation. (D) Schematic time schedule of the adapted DC infiltration protocol. Mature DCs and skin samples were cocultured. After 24 hours the samples were washed and culturing was extended for another 24 hours (with a total assay time of 48 hours) or 72 hours (with a total assay time of 96 hours) in standard culturing medium. (E) GVHR scoring of the samples following the adapted DC infiltration protocol executed as depicted in D at various time points. (F) Detailed analysis of the GVHR background at various time points using 0.125 x 10⁶ mDC following the protocol depicted in D. In all cases, GVHR scores were grade II or lower. (G) Mature DC infiltration after 96 hours with 0.125 x 10⁶ mDC. The arrows indicate CFDA-SE-labeled DCs.
Skin GVHD grading

The histopathologic grading of formalin-fixed and paraffin-embedded skin explants was assessed and confirmed independently. Skin GVHR was scored according to the scoring system of Lerner\(^2\)\(^1\). The grading system was as follows: grade 0, normal skin; grade I, mild vacuolization of epidermal basal cells; grade II, diffuse vacuolization of basal cells with scattered dyskeratotic bodies; grade III, subepidermal cleft formation; and grade IV, complete epidermal separation. Because incubation with autologous PBMC or medium alone can result in GVHR grades 0, I, and II\(^3\) (Figure 1C), these scores were considered as background.

Detection of HA-1 CTL activation on cytocentrifuged preparations

For detection of infiltrated and activated HA-1-specific CTLs upon coculture with HA-1-positive or -negative EBV-BLCL, a triple-staining method was used combining CD3, CD20, and either Granzyme B or Ki67. Acetone-fixed cytocentrifuge preparations were preincubated for 30 minutes in 5% (v/v) normal goat serum (Sanquin) followed by incubation with mouse anti-CD20 monoclonal antibody (mab), biotinylated goat anti mouse, Streptavidin-conjugated Cy5, normal mouse serum (Sanquin), PE-conjugated mouse anti-Granzyme B mab, rabbit anti-PE, Cy3-conjugated goat anti rabbit, normal mouse serum, and FITC conjugated anti-CD3. For combination with Ki67, incubation in normal goat serum was followed by rabbit anti-Ki67 antibody, mouse anti-CD20 mab, biotinylated goat anti mouse, Cy3-conjugated goat anti rabbit antibody, Streptavidin-conjugated Cy5, normal mouse serum, and FITC-conjugated anti-CD3.

Detection of HA-1-specific CTLs by tetrameric HLA-A2/HA-1 peptide complexes by multicolor staining

For multicolor fluorescence staining of cryopreserved skin tissues, a fluorescence staining protocol was used as described previously with minor modifications\(^2\)\(^2\). In brief, skin explant tissues preincubated with mature DCs overnight and HA-1-specific CTLs for 72 hours were labeled with APC-conjugated HLA-A2/HA-1 tetramer (10µg/mL) overnight at 4°C with gentle shaking. After washing 3 times with PBS, the skin explant tissues were cryopreserved. Acetone-fixed cryosections were preincubated for 30 minutes in 5% (v/v) normal mouse serum (Sanquin) and subsequently incubated for 30 minutes in goat anti-APC antibody
(Biogenesis), Alexa 633-conjugated donkey antigoat antibody (Molecular Probes Europe BV, Leiden, The Netherlands), PE-conjugated mouse anti-Granzyme B antibody (Sanquin), rabbit anti-PE (Biogenesis), normal goat serum (Sanquin), Cy3-conjugated goat antirabbit (Jackson Immuno Research Laboratories, Inc.), and FITC-conjugated mouse anti-CD8 antibody (Becton and Dickinson, San Jose, CA). Sections were mounted on glass slides, covered, and analyzed by CLSM.

**CLSM analysis and scoring of HA-1-specific CTL infiltration**

CD8-FITC, Granzyme B-PE (Alexa 568 or Cy3) and HLA-A2/HA-1 tetramer-APC (Alexa 633 or Cy5) signals were collected separately on a Leica TCS SP confocal system (Leica Microsystems, Heidelberg, Germany) equipped with an Argon-Krypton-Helium/Neon laser. Images were taken sequentially using a x40 numerical aperture 1.4 objective. Color photomicrographs were generated as electronic overlays. The skin sections were scanned in X-, Y-, and Z-axis directions to obtain a 3-dimensional confocal image window. Per confocal image window, quantitative analysis was performed in the focal plane with maximum infiltration of cells. Cells that were triple positive for HLA-A2/HA-1 tetramer, CD8, and Granzyme B were counted according to the following scoring system: negative = 0 or 1 triple positive cell; intermediate = 2 or 3 triple positive cells; positive = more than 4 triple positive cells.

**RESULTS**

**Mature but not immature DCs reconstitute ex vivo skin explants without damaging the skin tissues**

Because the goal of our study was to investigate the presence of hematopoietic HA-1⁺ APCs on the subsequent development of GVHR upon addition of HA-1-specific CTLs, we first studied the influence of the maturation status of DCs and the numbers of added DCs on the skin explants samples. Hereto, skin biopsies were coincubated with 0.5 x 10⁶ CFDA-SE labeled mature or immature DCs for 2, 24, 48, or 72 hours, as schematically depicted in Figure 1A. CFDA-SE positive cells were observed in the skin biopsies incubated with matured DCs but not in those incubated with immature DCs (Supplementary Figure S1). Thus, only mature DCs infiltrated the skin samples. Moreover, these mature DCs displayed
phenotypic characteristics resembling the phenotype of dermal DCs, that is, a high expression of CD1a, HLA-DR, and CD86, and absence of CD14. Consequently, all subsequent experiments were performed with these mature DCs.

To investigate whether reconstitution with mature DCs alone might cause skin tissue damage in our model, skin samples were incubated with $0.5 \times 10^6$ mature DCs for 2, 24, 48, and 72 hours. Figure 1A, B, and C indeed shows that addition of mature DCs results in GVHR score III after 24 hours. Reduction to background GVHR was obtained by extensive washing after DC incubation (Figure 1D) and by applying lower doses of mature DCs ($0.125 \times 10^6$, $0.25 \times 10^6$, and $0.5 \times 10^6$). Using this adapted skin DC infiltration protocol, all GVHR scores determined after 24 hours and 96 hours were below background (Figure 1E). Thus, because DC infiltration could be established with $0.125 \times 10^6$ DCs (Figure 1F) without causing significant GVHR (Figure 1E), all subsequent experiments were performed with the latter number of DCs.

Supplementary Figure S1. Influx of mature and immature DCs in skin explant tissues. CFDA-SE-labeled mature and immature DCs were incubated with skin explant tissues as indicated. The arrows indicate infiltrated CFDA-SE-labeled DCs.
Figure 2. Activation of HA-1-specific CTLs depends upon recognition of their cognate antigen. HA-1 specific CTLs were cocultured with HA-1-positive (A, C) or HA-1-negative (B, D) EBV-BLCL. Activation was monitored by immunofluorescent triple-color staining on cytocentrifuge preparations. EBV-LCL were visualized with anti-CD20 (blue) and T cells with anti-CD3 (green) in combination with either activated cytotoxic cell marker Granzyme B (red; A and B) or nuclear activation marker Ki67 (red; C and D). Activated and unactivated HA-1-specific CTLs have been marked by red and white arrows, respectively. Original magnification is x 400; scale bar represents 10µm.

Infiltration and activation of HA-1-specific CTLs depends on the presence of mature DCs in HLA-A2/HA-1+ Skin
To verify the activation potential of CTLs, HA-1-specific CTLs were coincubated with HA-1+ and HA-1- EBV-BLCLs in vitro, cytocentrifuged, and stained with the proliferation marker Ki-67 and the cytotoxicity marker granzyme B. Only upon recognition of their cognate antigen, HA-1-specific CTLs acquired expression of Ki-67 and displayed an eccentrically, polarized cytoplasmic expression pattern of granzyme B (Figure 2), indicating their activation. Subsequently, infiltration and activation of HA-1-specific CTLs were analyzed in the skin samples reconstituted with various HLA- and HA-1-types of mature DCs. Hereto, skin samples of donors with different HLA- and HA-1-types (HLA-A2+/− and/or HA-1+/−) were
preincubated with DCs of different HLA- and HA-1-types (HLA-A2+/− and/or HA-1+/−) for 24 hours (Table 1). After washing, the skin samples were coincubated with the HLA-A2+ HA-1-specific CTLs for 3 days and scored for CTL infiltration and activation using CLSM analysis. Skin samples were considered to be infiltrated by activated CTLs if CTLs triple stained for HA-1 tetramer/CD8/granzyme B. Infiltrated activated HA-1-specific CTLs were observed in all skin biopsies pre-incubated with HLA-A2+/HA-1+ mature DCs (Figure 3 and Table 1). Moreover, infiltrated activated HA-1-specific CTLs were also present in the skin of HLA-A2+/HA-1+ individuals, independent of the HLA- and HA-1 phenotype of the mature DCs. Interestingly, infiltrated activated HA-1-specific CTLs were also observed in HLA-A2+/HA-1− skin in the presence of HLA-A2+/HA-1+ mature DCs. Notably, all infiltrated CTLs were activated. CTLs were neither detectable in HLA-A2− skin, nor in combinations where DCs and the skin were both HA-1−. Thus, infiltration and activation of HA-1-specific CTLs depends on the HLA-A2/HA-1 phenotype, expressed either by the exogenously added DCs alone or by a combination of skin and DCs.

Table 1. Infiltration of activated HA-1-specific CTLs in skin explants depends on HLA- and HA-1-phenotype of the skin and the DC

<table>
<thead>
<tr>
<th>mDCs</th>
<th>Medium</th>
<th>A2+/HA-1+</th>
<th>A2+/HA-1-</th>
<th>A2-/HA-1+</th>
<th>A2-/HA-1-</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>3/3</td>
<td>5/5</td>
<td>5/5</td>
<td>3/3</td>
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<td>3/3</td>
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</tr>
<tr>
<td>A2-/HA-1-</td>
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</table>

HA-1-specific cytotoxic T cells (CTLs) and dendritic cells (DCs) were induced to infiltrate skin explant tissues according to the adapted skin infiltration protocol as described in Materials and Methods. Granzyme B positivity was defined as a polarized intracellular staining pattern. Activated infiltrated CTLs were scored as HA-1 tetramer/CD8/granzyme B triple-positive cells by confocal laser scanning microscopy (CLSM) analysis. Results are depicted as number of samples with positive score per total number of samples tested.
Figure 3. Infiltration and activation of HA-1-specific CTLs detected by *in situ* combined tetramer staining. HA-1 CTLs were cocultured with DC preincubated skin explants according to the adapted skin infiltration protocol described in Materials and Methods. Activated HA-1-specific CTLs (yellow arrows) were identified by triple-color staining for HLA-A2/HA-1 tetramer (blue), CD8 (green), and polarized Granzyme B (red). Scale bar represents 10µm.

HA-1-specific CTLs do not cause severe GVHR in the *ex vivo* skin explant model

In most combinations, HA-1-specific CTLs that infiltrated the skin did not cause severe GVHR in DC reconstituted skin samples (Table 2). Only in 2 specific combinations was GVHR of grade III or IV observed; that is, HLA-A2+/HA-1+ mature DCs in HLA-A2+/HA-1+ skin (1 of 3 individuals) and HLA-A2+/HA-1+ mature DCs in HLA-A2+/HA-1+ skin (1 of 6 individuals; Table 2). CLSM analysis of these skin sections revealed granzyme B-expressing HA-1-specific CTLs in the dermis at the dermal/epidermal junction and around the edges of the GVHR lesions of HLA-A2+/HA-1+ skin incubated with A2+/HA-1+ mDCs. Thus, HA-1-specific CTLs can infiltrate skin tissue where they get activated; these T cells, however,
appear, in 7 of the 9 combinations, not to be able to induce GVHR in our *ex vivo* skin explant model. Positive control experiments using HLA-A2⁺ female (HY⁻) skin preincubated with HLA-A2⁺ male (HY⁺) mature DCs resulted in GVHR grade III after 3 days incubation with HLA-A2/HY-specific T cells, indicating that collateral damage can be measured in our adapted skin explant model (Supplementary Figure S3).

**Table 2. HA-1-specific CTLs do not induce severe GVHR in the skin explant model**

<table>
<thead>
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<th>mDCs</th>
<th>Medium</th>
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<th>A2⁺/HA-1⁻</th>
<th>A2⁻/HA-1⁺</th>
<th>A2⁻/HA-1⁻</th>
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</tr>
<tr>
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</tr>
</tbody>
</table>

HA-1 cytotoxic T cells (CTLs) and dendritic cells (DCs) were induced to infiltrate skin explant tissues according to the adapted skin infiltration protocol as described in Materials and Methods. Results are depicted as number of samples with positive graft-versus-host reactivity (GVHR) score (grade III or IV) per total number of samples tested. Positive control experiments using HLA-A2⁺ female (HY⁻) skin with HLA-A2⁺ male (HY⁺) mature DCs results in GVHR grade III after 3 days incubation with HLA-A2/HY-specific T cells, indicating that collateral damage can be measured in our adapted skin explant model.

**Supplementary Figure S2.** CD1a-positive cells in explant tissues. (A) Directly snap-frozen skin sample (green = CD8, red = CD1a). (B) Skin incubated O/N with medium, washed, and incubated for 72 hours. (C) Skin incubated O/N with allogenic DC, washed, and incubated for 72 hours. White arrows indicate CD1a-positive cells.
Supplementary Figure S2. CD1a-positive cells in explant tissues. (A) Control experiment with female (HY−) skin preincubated with HLA-A2+ female (HY−) mature DCs and subsequent incubation with HLA-A2/HY-specific T cells overnight. (B) Control experiment with female (HY−) skin preincubated with HLA-A2+ male (HY+) mature DCs and subsequent incubation with HLA-A2/HY-specific T cells overnight (green signal = CFDA-SE, blue signal = HLA-A2/HY tetramer, red signal = Ki67). Green arrows indicate the infiltrated mature DCs, white arrows mark HLA-A2/HY-specific T cells, and red arrows point at activated T cells.

DISCUSSION

Several studies reported the influence of the minor H antigen HA-1 on the development of GVHD after HLA-matched SCT7–9. As yet, these data are poorly understood because the minor H antigen HA-1 is hematopoietic system-specific and neither functional studies nor mRNA analyses show expression of HA-1 on nonhematopoietic cells including melanocytes, keratinocytes, and fibroblasts23,24. Additionally, HA-1-specific CTLs neither infiltrated nor destroyed skin tissue in an ex vivo in situ skin explant model, despite the fact that the skin donors were HLA-A2+/HA-1+. Based on these previous experimental data, it is unlikely that mismatches in hematopoietic system specific miHAs, like HA-1, are involved in GVHD. However, the previous studies were either performed on cultured cell lines or on ex vivo skin tissues devoid of minor H antigen expressing APCs. Therefore, those studies could not address mechanisms like cross presentation of APC-derived HA-1 on nonhematopoietic skin cells or collateral damage of skin tissue as a result of the interaction between HA-1-specific T
cells and residual APCs in the skin. To address these questions, we adapted the skin explant assay by reconstituting the skin tissues with professional APCs. This step appeared to be essential, as the number of endogenous dermal DCs decreases during the skin explant assay (Supplementary Figure 2). We here show that the association of HA-1 with the development of clinical GVHD after HLA-matched minor H antigen HA-1 mismatched SCT may be explained by the presence of recipient’s APCs in the GVHD target organs. Namely, upon coincubation with HLA-A2+/HA-1+ DCs, skin infiltrated HA-1-specific CTLs express the activated CTL marker Granzyme B. However, those CTLs were not able to cause GVHR grade III/IV, whereas HLA-A2/HY T cells do (Supplementary Figure S3). This observation is potentially because of lower infiltrations rates of the HA-1-specific T cell clone or a lower lytic capacity. Limitation in the time frame of the skin explant model hampers extension of the incubation time to overcome such potential problems; GVHR in control samples raise to grade III/IV after 96 hours.

Residual host APCs in the skin may play an important role in the immune pathogenesis of skin GVHD. Merad et al. demonstrated in mice studies that depletion of host-derived residual APCs cells, prevents skin GVHD. The level of donor chimerism increased with the dose of coadministered allogeneic T cells. Apparently, these T cells are able to remove recipient APCs cells efficiently. This allore cognition of residual host APCs - which could potentially result from the cognate interaction between HA-1+ recipient derived APCs in the skin and donor-derived HA-1-specific T cells - may cause local GVHD. This effect can result from direct or indirect destructed skin tissue.

Indirect destruction can be the result of collateral damage. Collateral damage has been studied in the context of hepatitis. Serious hepatitis can result from collateral damage caused by virus-specific CTLs in influenza infection. Our data suggest that collateral damage may not be active in HA-1-mediated GVHD. Namely, HA-1-specific CTLs recognize the residual hematopoietic-restricted minor H antigen HA-1 presenting DCs, whereas no infiltration occurred in HLA-A2-skin or in HA-1-DC/skin combinations. Surprisingly, HA-1-specific infiltrated and activated T cells were not causing GVHR in the skin reconstituted with HLA-A2+HA-1+ DC, which could potentially be because of the phenotype of the HA-1-specific CTLs. A previous report suggests that perforin-granzyme B mediated lysis appeared to be restricted by direct allo-recognition, while FasL-mediated lysis is responsible for collateral
damage to self cells during an allogeneic response\textsuperscript{26}. The HA-1-specific T cells used in the present study express perforin-granzyme B.

Direct destruction of GVHD target tissues that do not express the HA-1 antigen requires transfer of HA-1 antigen from hematopoietic cells to the nonhematopoietic cells. Various mechanisms for antigen transfer have been described, including cellular acquisition of MHC/antigen complexes via exosome-resembling microvesicles\textsuperscript{27}. These microvesicles are involved in the functional transfer of MHC/antigen complexes to follicular DCs and between different DCs\textsuperscript{28}. They thereby act as important vehicles for spreading of antigen-specific signals. Similarly, these vehicles can transfer membrane-bound MHC/antigen complexes from T cells to APCs\textsuperscript{29}. Because we also observed infiltration and activation of HA-1-specific T cells in HLA-A2\(^+\), HA-1\(^-\) skin reconstituted with HLA-A2\(^+\), HA-1\(^+\) DCs, we cannot fully exclude transfer of membrane bound MHC/antigen complexes as a mechanism. It is, however, likely that in the particular combination of HLA-A2\(^+\), HA-1\(^+\) DCs in HLA-A2\(^+\)HA-1\(^-\) skin, the observed activation is because of a cognate interaction of the reconstituted DCs with the HA-1-specific T cells. Moreover, transfer of only HLA molecules is irrelevant in the HLA-identical SCT setting, as the transferred HLA molecules of donor origin are identical to those expressed by the recipient.

Neijssen et al.\textsuperscript{30} demonstrated that exchange of intracellular peptides can occur via gap junctions. Transfer through gap junctions applies to peptides with a relative molecular mass of up to approximately 1800. This intercellular peptide transfer results in CTL recognition of adjacent, innocent bystander cells. It is possible that in our system, antigen exchange between hematopoietic cells and nonhematopoietic cells may occur; the combination of HLA-A2\(^+\) HA-1\(^-\) skin reconstituted with HLA-A2\(^+\)HA-1\(^+\) DCs leads to activation of HA-1-specific T cells in the skin. The fact that T cell activation was observed in all DC reconstituted HLA-A2\(^+\)HA-1\(^+\) skin samples, indicates that also under normal conditions, nonhematopoietic tissues may acquire antigens from hematopoietic cells trafficking via the skin. In these combinations, the added mature DCs facilitate the infiltration of the HA-1-specific T cells into the skin, where the T cells can subsequently target nonhematopoietic skin cells that may have acquired the HA-1 antigen from autologous DCs prior to skin tissue sampling. The role of chemokines and chemokine receptors in the infiltration process is currently under investigation. In summary, we show that reconstitution of skin samples with mature DCs facilitates infiltration and
activation of HA-1-specific T cells. Although these CTLs were activated, they were not able to cause GVHR grade III/IV, possibly as a result of limitations of the model system. Moreover, our T cell activation data may imply that autologous residual minor H antigen HA-1-positive dermal DCs could potentially transfer their antigens to skin cells, leading to cognate antigen recognition, T cell activation, and destruction of the skin. These observations may help to understand the earlier reported involvement of HA-1 in the development of clinical GVHD.

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REFERENCES

HA-1 T cells in skin GVHD
