Concurrent Detection of Circulating Minor Histocompatibility Antigen-Specific CD8+ T Cells in SCT Recipients by Combinatorial Encoding MHC Multimers

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Abstract

Allogeneic stem cell transplantation (SCT) is a potentially curative treatment for patients with hematologic malignancies. Its therapeutic effect is largely dependent on recognition of minor histocompatibility antigens (MiHA) by donor-derived CD8+ T cells. Therefore, monitoring of multiple MiHA-specific CD8+ T cell responses may prove to be valuable for evaluating the efficacy of allogeneic SCT. In this study, we investigated the use of the combinatorial encoding MHC multimer technique to simultaneously detect MiHA-specific CD8+ T cells in peripheral blood of SCT recipients. Feasibility of this approach was demonstrated by applying dual-color encoding MHC multimers for a set of 10 known MiHA. Interestingly, single staining using a fluorochrome- and Qdot-based five-color combination showed comparable results to dual-color staining for most MiHA-specific CD8+ T cell responses. In addition, we determined the potential value of combinatorial encoding MHC multimers in MiHA identification. Therefore, a set of 75 candidate MiHA peptides was predicted from polymorphic genes with a hematopoietic expression profile and further selected for high and intermediate binding affinity for HLA-A2. Screening of a large cohort of SCT recipients resulted in the detection of dual-color encoded CD8+ T cells following MHC multimer-based T cell enrichment and short ex vivo expansion. Interestingly, candidate MiHA-specific CD8+ T cell responses for LAG3 and TLR10 derived polymorphic peptides could be confirmed by genotyping of the respective SNPs. These findings demonstrate the potency of the combinatorial MHC multimer approach in the monitoring of CD8+ T cell responses to known and potential MiHA in limited amounts of peripheral blood from allogeneic SCT recipients.

Introduction

In HLA-identical allogeneic stem cell transplantation (SCT), alloreactive CD8+ T cells specific for minor histocompatibility antigens (MiHA) play a pivotal role in graft rejection, graft-versus-host disease (GVHD) and the curative graft-versus-tumor (GVT) response. Several MiHA have been molecularly defined with the potential to induce a GVT response without inducing GVHD, such as HA-1 [1–4], LRH-1 [5] and ACC-1 [6]. Although MiHA can be regarded as the most dominant antigens in GVT immunity, the CD8+ T cell response rate towards these antigens has not been followed extensively in transplanted patients. Furthermore, most analysis focused on the detection of CD8+ T cell responses to single MiHA epitopes using conventional techniques such as single-tetramer staining or the ELISPOT assay.

Fluorescent labeled peptide-major histocompatibility antigen (MHC) complexes, known as MHC multimers, are excellent reagents to monitor MiHA-specific T cell responses after SCT and donor lymphocyte infusion (DLI) in peripheral blood of transplanted patients. Especially, the recently developed combinatorial encoding technique using dual-color encoded MHC multimers is a very attractive approach to accurately detect multiple MiHA-specific T cells in one sample [7]. The principle of this method relies on the flow cytometric detection of a single T cell population that is stained with different fluorochrome-labeled MHC multimers. This dual-color encoded MHC multimer approach has the ability to detect up to 15 different T cell populations when using 6 different fluorochromes [7]. Therefore, a key advantage compared to single-tetramer staining is that the amount of patient peripheral blood cells needed is equal to just one labeling, making the technique very suitable when dealing with limited amounts of patient material. The combinatorial encoding approach can accommodate a wide range of different peptide-MHC multimers for several HLA molecules, which can be readily produced through UV-mediated ligand exchange [8,9]. The versatility of these two methods makes the combinatorial encoding MHC multimer technique an excellent monitoring tool for detecting MiHA-specific CD8+ T cell responses against a panel of known MiHA.
Further, another potential application of the combinatorial encoding MHC multimer approach could be its use to identify new MiHA. Recently, the value of the method for antigen discovery has been demonstrated for the identification of melanoma-associated T cell epitopes [7]. Here, we explored the use of the combinatorial MHC multimer technique for the detection of CD8\(^{+}\) T cell responses in transplanted patients against candidate MiHA defined through a reverse immunology approach. Interestingly, we detected peptide-specific dual-tetramer positive CD8\(^{+}\) T cells against 8 out of 75 HLA-A2 binding peptides that were predicted in silico from polymorphic hematopoietic-specific genes.

Collectively, our results illustrate that the combinatorial MHC multimer method is a suitable technique to analyze patients after SCT and DLI for the concurrent occurrence of MiHA-specific CD8\(^{+}\) T cells targeting known MiHA or candidate MiHA identified by reverse immunology approaches.

Results

Immunomonitoring of MiHA-specific CD8\(^{+}\) T cell responses using combinatorial MHC multimer staining

The success rate of immunological responses in patients post-SCT and DLI can be assessed by measuring the MiHA-specific T cells present in the blood of the patient [1,2,5]. Since patient material is often in short supply, we developed a technique that can screen for multiple MiHA-specific T cells in a limited amount of patient follow-up material. This technique makes use of dual-color-encoded MHC multimers to detect multiple antigen-specific T cells in one sample [7,10]. In order to set up such a MiHA-multimer kit, we have chosen a set of 10 MiHA, each being encoded by two different fluorochromes coupled to an MHC multimer containing an individual peptide (Table 1).

To determine whether the developed MiHA-multimer kit is suitable for the detection of multiple MiHA-specific T cells in blood samples of transplanted patients, we selected a cohort of patients transplanted with partially T-cell depleted stem cell grafts from HLA-identical sibling donors. These patients also received pre-emptive DLI if they did not develop significant GVHD after SCT. These patients and their respective donor were typed for MiHA mismatches using fluorescence-based competitive allele specific PCR (Table 2). The selected SCT recipients were mismatched for one or two MiHA with their HLA-identical donor and no tetramer\(^{+}\) T cells were observed in pre-SCT recipient PBMC (Table 2). For the disparate MiHA a dual-color tetramer analysis was performed using both APC and PE labeled tetramers (Figure 1A). Since double positive events were scored, the dual-color tetramer stainings result in accurate detection of low frequency MiHA-specific CD8\(^{+}\) T cells [10], even at relatively late time points post-SCT. Specific detection of these low frequency MiHA-specific CD8\(^{+}\) T cell populations was verified after in vitro stimulation of peripheral blood mononuclear cells (PBMC) samples with MiHA peptide-pulsed EBV-LCL (Figure 1A).

Knowing the MiHA-specific T cell status of the patient samples, we tested a second cryopreserved PBMC sample again by the new five-color combinatorial encoding MiHA-multimer kit (Figure 1B). Results of a representative patient (i.e. UPN348) can be seen in Figure 2A. For this patient’s cryopreserved PBMC sample at 31 weeks post-SCT, we observed 0.11% HY-B7-tetramer\(^{+}\) T cells using dual-color staining (Figure 1A), compared to 0.04% by the five-color MiHA-multimer kit (Figure 1B and 2A). Specific detection of CD8\(^{+}\)tetramer\(^{+}\) T cells was verified after in vitro stimulation of PBMC samples with HY-B7 peptide-pulsed EBV-LCL, showing 0.17% HY-B7-tetramer\(^{+}\) cells with the MHC-multimer kit (Figure 2B) compared to 0.15% by dual-color staining (Figure 1A). All different MiHA-specific CD8\(^{+}\) T cells found by dual-color staining could also be found in the thawed PBMC samples analyzed by the new five-color combinatorial encoded MiHA-multimer assay, although the frequency of MiHA-specific cells was somewhat lower (Table 2). These data demonstrate that circulating MiHA-specific CD8\(^{+}\) T cells can be detected concurrently using a combinatorial encoding MiHA-multimer kit, making this a valuable tool for immunomonitoring of SCT recipients using a limited amount of patient material.

Identification of novel candidate MiHA by reverse immunology

Next, we studied the feasibility of the combinatorial encoding MHC-multimers as an identification tool for the discovery of novel MiHA. In order to predict HLA-A2-restricted candidate MiHA within hematopoietically restricted gene products, we developed and tested an adaptable computer algorithm. An arbitrary minimum predicted half-time of dissociation (T\(_{1/2}\)) of 30 seconds was set for candidate HLA-A2 restricted MiHA, based on corroboration with known HLA-A2 peptide and MiHA binding affinities, which indicated that a peptide with moderate HLA-A2 binding affinity would exhibit a predicted T\(_{1/2}\) of >30 seconds. Subsequently, candidate MiHA were further selected, based upon either observed allele frequencies in Caucasians or allele frequencies reported by dbSNP/HapMap. Typically, only those candidate MiHA that had a reported allele frequency for the candidate MiHA-positive allele between 15% and 46% were pursued as candidate MiHA, since MiHA with frequencies within this range yield a theoretical clinical applicability of more than 20% in HLA-A2 matched unrelated recipient-donor pairs.

Peptides of the predicted candidate MiHA were synthesized and HLA-A2 monomers were generated using the UV-mediated ligand exchange method [9]. All monomers were tested for stable binding of the predicted epitope using an ELISA-based HLA-affinity assay [9], which revealed that 75 out of 107 predicted potential MiHA bind HLA-A2 with high (OD\(\geq\)1.25) or intermediate (OD 0.4–1.24) affinity (Figure 3). As a positive control for successful peptide exchange, viral (EBV, CMV, FLU) HLA-A2 specific peptides were used, as well as negative controls of not applying UV light or not adding any peptide at all (data not shown). Peptides with low affinity to HLA-A2 (OD\(<\)0.4) were discarded from further experiments and only HMC-monomers
Table 2. Characteristics of SCT recipients containing circulating MiHA-specific CD8+ T cells.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>Sex (Rt/Do)</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>DNA MiHA mismatch</th>
<th>Tetramer+ cells by dual color Pre-SCT</th>
<th>Screened material - Time post SCT (wks)</th>
<th>Tetramer+ cells Dual color vs five color*</th>
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<tbody>
<tr>
<td>509</td>
<td>NHL</td>
<td>M/F</td>
<td>1</td>
<td>2</td>
<td>8 51</td>
<td>HA-8</td>
<td>30 post SCT &lt;0.01%</td>
<td>30 post SCT &lt;0.01% &lt;0.01%</td>
</tr>
<tr>
<td>348</td>
<td>CML</td>
<td>M/F</td>
<td>1</td>
<td>11</td>
<td>7 8</td>
<td>HY</td>
<td>31 post SCT &lt;0.01%</td>
<td>31 post SCT 0.11% 0.04%</td>
</tr>
<tr>
<td>438</td>
<td>NHL</td>
<td>F/M</td>
<td>2</td>
<td>31</td>
<td>51</td>
<td>HA-1</td>
<td>No material</td>
<td>62 post SCT 0.02% 0.02%</td>
</tr>
<tr>
<td>8605</td>
<td>MM</td>
<td>F/M</td>
<td>2</td>
<td>11</td>
<td>51 35</td>
<td>ACC-1</td>
<td>74 post SCT &lt;0.01%</td>
<td>74 post SCT &lt;0.01% &lt;0.01%</td>
</tr>
<tr>
<td>315</td>
<td>AML</td>
<td>M/M</td>
<td>3</td>
<td>24</td>
<td>7 35</td>
<td>PANE1</td>
<td>68 post SCT &lt;0.01%</td>
<td>68 post SCT 1.28% 0.38%</td>
</tr>
<tr>
<td>265</td>
<td>AML</td>
<td>M/F</td>
<td>2</td>
<td>3</td>
<td>38 47</td>
<td>HY</td>
<td>No material</td>
<td>24 post SCT 0.04% 0.04%</td>
</tr>
</tbody>
</table>

*Statistical analysis between dual-color and five-color tetramer analysis was performed using the unpaired t test. The p value is 0.2903.

Abbreviations: Rt, recipient; Do, donor; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma.

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Concurrent detection of multiple peptide-specific CD8+ T cells after enrichment and expansion

To investigate whether peptide-specific CD8+ T cells could be found for the predicted MiHA epitopes that had high or intermediate affinity to HLA-A2, a cohort of 31 patients was selected for screening of the follow-up material collected after DLI. Approximately half of the patients selected, developed acute GVHD after SCT and/or acute or chronic GVHD after DLI. These observations show the presence of an allo-response that might have been mediated by MiHA-specific T cells making these patients attractive to look for candidate MiHA. To detect epitope-specific CD8+ T cells in the patient PBMC collected after DLI (average 18 weeks post DLI) we first enriched the samples using PE-labeled MHC multimers. Figure 4 shows an example of an enriched culture containing low frequency CD8+ T cells specific for the HLA-A2-restricted EBV (GLCTLVAML) and CMV (NLVPVMATV) epitopes (respectively 0.03% and 0.38%) pre-culture. MHC-multimer-based enrichment and subsequent expansion yielded a CD8+ T cell population consisting of 0.85% EBV-TET+ and 11.5% CMV-TET+ cells after 16 days of culturing. This shows the potential of this method to increase low frequency T cells to sufficient cell numbers of multiple specificities for further experiments.

To detect multiple MiHA peptide-specific CD8+ T cells against our pre-selected panel of 75 candidate HLA-A2-restricted peptides, we performed enrichments on 31 different patient samples of which 17 resulted in sufficient cell numbers for analysis. We detected peptide-specific CD8+ T cells in cultures of 5 different patients (Table 3). Notably, we detected a CD8+ T cell response against the known MiHA SMCY-A2. Additional patients were transplanted with donor grafts mismatched for any of the control HLA-A2 MiHA included in the analysis as was determined by SNP genotyping. However, we did not observe specific T-cell responses for these MiHA. Results of a representative patient (UPN665) are shown in Figure 5A. PBMC from this patient obtained at 14 weeks post DLI were enriched, expanded and analyzed for all 6 combinatorial MHC-multimer encoding pools. In pool A, two specific T cell populations were found. The combination of miHAs coupled to APC and Qdot705 showed a positive response of 0.55% dual-color tetramer positive T cells, recognizing the WT-1-derived epitope RMFPNAPYL. In addition, the combination of miHAs coupled to Qdot605 and Qdot655 showed a positive response of 0.07% dual-color tetramer positive T cells, recognizing the BPI-derived epitope KLQPYFQTL.

To verify whether the observed T cell responses correlate with SNP mismatches at the DNA level, we performed allele specific genotyping of donor and recipient for the SNPs corresponding to the immunogenic epitopes found in the combinatorial MHC-multimer staining. Although several mismatched SNPs between donor and recipient were found, only 2 out of 8 epitopes found in the screening corresponded with their respective SNP genotype (underlined in Table 3). These two candidate MiHA (i.e. LAG3 derived LTLGVLSLL and CARD100 derived YLVPSDALL), as well as epitopes to which a high response was observed (epitopes derived from WT-1 and TLR10), were selected for further experiments. However, dual-color tetramer stainings using PE- and APC-conjugated tetramers could not confirm specific CD8+ T cells for the found peptides in non-cultured PBMC. Although we were not able to verify responses found in the analysis, the use of combinatorial encoding MHC multimer panels proved useful for high throughput concurrent detection of specific CD8+ T cells against a large panel of candidate MiHA peptides.

Discussion

The monitoring of multiple MiHA-specific T cells in vivo can give important additional information on the clinical response and might lead to more insight into the therapeutic applicability of specific MiHA. In this study, we report a novel approach for the monitoring of MiHA-specific T cells in a limited number of PBMC from patients after SCT and DLI, which we believe is of great value for the immunomonitoring of SCT recipients. Cryopreserved PBMC samples from patients who underwent partially T cell depleted allogeneic SCT, in some cases followed by DLI, were simultaneously analyzed for 10 different MiHA-specific T cells. In all 6 patients analyzed with the set of combinatorial encoded MHC multimers, MiHA-specific T cells could be detected. These
Concurrent Detection of MiHA-Specific CD8+ T Cells

A  Dual-color combination
PBMC sample  Post peptide stimulation

- UPN509
  - PBMC sample: 0.16%
  - Post peptide stimulation: 0.88%

- UPN348
  - PBMC sample: 0.11%
  - Post peptide stimulation: 0.15%

- UPN438
  - PBMC sample: <0.01%
  - Post peptide stimulation: 0.17%

- UPN8605
  - PBMC sample: 0.02%
  - Post peptide stimulation: 2.16%

- UPN315
  - PBMC sample: 0.04%
  - Post peptide stimulation: 0.22%

- UPN735
  - PBMC sample: 0.04%
  - Post peptide stimulation: 0.97%

B  Five-color combination
PBMC sample

- HYA2
  - PBMC sample: 0.09%

- HYB7
  - PBMC sample: 0.04%

- HA-1
  - PBMC sample: 0.02%

- HA-1
  - PBMC sample: 0.01%

- ACC-1
  - PBMC sample: 0.07%

- HA-8
  - PBMC sample: 0.04%

- PANE1
  - PBMC sample: 0.38%
MiHA-specific T cells proliferated upon stimulation with MiHA-peptide, showing a peptide-dependent response. To confirm functionality of the MiHA-specific T cells, proliferation upon stimulation with endogenously processed peptides should be measured. The specificity of the cells found in the analysis corresponded to the results obtained by conventional T cell screenings. The use of a combinatorial MiHA-multimer kit gives a quick overall view of numerous MiHA-specific T cells within patient blood samples post SCT. Moreover the amount of patient material used is equal to what conventional screenings use for just one specific staining. This method already has been applied as a tool for the simultaneous detection of autoreactive CD8\(^+\) T cells in blood [11] and now we show that it is a valuable tool for the concurrent detection of multiple MiHA-specific T cells after allogeneic SCT and DLI. The MiHA-detection kit used in this study already covers 10 prevalent MiHA, though flexibility of the technique allows further expansion of the MiHA set. However, the current technical limitations regarding the synthesis of HLA-monomers prevent implementation of certain MiHA, such as MiHA presented in HLA-B44. The MiHA-detection kit can be expanded by multimers coupled to additional fluorophores such as Qdot800 or Qdot565 and Qdot585. It should be noted that these three fluorophores have a low intensity for which should be corrected when combining with other fluorophores [7].

Because of the techniques' potential to screen for large numbers of different specific T cells [11] and its success in the identification of melanoma-associated T cell epitopes [7], we tested the potential of the combinatorial encoding approach to identify new MiHA that were predicted from a set of polymorphic genes expressed by the hematopoietic system. For the development of adjuvant tumor-specific immunotherapy after SCT it is important to enlarge the spectrum of molecularly identified MiHA that are selectively expressed in the hematopoietic system and in hematologic malignancies. Investing in new high throughput methods for MiHA identification is in our opinion critically important. The recently developed technique using dual-color-encoded MHC multimers to detect multiple antigen specific T cells in one sample [7] was tested for this purpose. The combinatorial encoding approach already led to the discovery of a new melanoma-associated epitope in a screening of T cells [7], showing its applicability in these kind of studies. Our present article describes the prediction of MiHA from genes with a hematopoietic restricted expression profile and the verification of these MiHA by screening for MiHA specific CD8\(^+\) T cells in patient PBMC following MHC multimer-based enrichment and a short-term expansion. A similar reverse immunology approach of predicting MiHA has already yielded a set of novel MiHA originating from the Y chromosome presented in HLA-A2 [12]. These male-specific MiHA were verified by means of ELISPOT showing some of these MiHA to be even more immunogenic than the well known SMCY-A2 male specific MiHA. Although this indicates that predicting MiHA can yield actual functional MiHA, this set of MiHA is not of therapeutic interest since it is not to be expected that these MiHA have a hematopoietically restricted expression profile.

Here we performed an analysis using combinatorial encoded multimers specific for 75 different epitopes. Of the 31 enriched cultures we screened 17 and found multimer-positive T cells in 5 of the cultures. One of the specific T cell populations found was directed against the tumor associated antigen WT-1 for which specific T cells can be found in CML [13], MDS and AML patients [14,15]. In the set of predicted candidate MiHA, the gene WT-1 was integrated because of the presence of SNP rs9332973 resulting in the substitution of a threonin for the alanin at position 6 of the nonamer epitope. Multimers were made for both epitopes since they had high affinity to HLA-A2, however only the well known WT-1 epitope yielded an actual T cell response (0.55%) in patient UPN655 who was diagnosed with AML. Another epitope to which a high number of CD8\(^+\) T cells could be cross reactive between these two epitopes since the alternative amino acid is not present at the anchor side to the HLA molecule. Though several specific responses were detected in the screening of the enriched patient PBMC, none of these responses could be validated by conventional tetramer staining or culture methods. Known HLA-A2-restricted MiHA (i.e. HA-1, HA-2, HA-8 and SMCY) were added to the screening panel. However, we only found 1 response for the control MiHA SMCY-A2 (Table 3). DNA of all patients and their corresponding donors used in the screening was genotyped for the control MiHA. Mismatches for these MiHA were detected in 6 out of 17 screened patients. Conventional tetramer staining of the PBMC of these patients followed by tetramer validation after stimulation with peptide-pulsed EBV-LCL revealed MiHA T cell responses in 4 out of the 6 mismatched patients of which 3 were not found by screening with the combinatorial encoding set.

A potential benefit of our approach is that the epitopes were predicted from a set of genes with a restricted expression pattern, which gives an advantage when looking for tissue restricted MiHA. However, there are critical limitations of our algorithmic model and selection criteria for novel candidate MiHA. While the majority of MiHA identified thus far are derived from allelic SNPs that generate an amino acid disparity (i.e., nonsynonymous SNPs) between donor and recipient, other variables may mask the presence of MiHA or otherwise confound such analyses. Many MiHA are likely to be encoded by polymorphic cDNAs or ESTs that are simply not yet represented in GenBank as translated polypeptides, such as protein isoforms derived from relatively obscure alternative splicing variants [18], cryptic translation products [19–21], or perhaps transposable elements (A.G.B., manuscript in preparation). Another possibility is that even though a MiHA-encoding gene is represented in GenBank, its relevant polymorphisms may not yet be fully characterized. Further complicating the algorithmic prediction of MiHA peptides is the demonstration that in certain instances, peptides can be excised and spliced in the proteasome prior to presentation by MHC [22,23]. Another important caveat is that the selection of T cells specific for candidate antigens with

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**Figure 1. Detection of MiHA-specific CD8\(^+\) T cells in peripheral blood of patients after SCT and DLI.** (A) PBMC collected post SCT stained with PE- and APC-conjugated tetramers, CD8 AlexaFluor 700, CD4-, CD14-, CD16- and CD19-FITC and Sytox Blue. The remaining PBMCs were stimulated once with the MiHA peptide pulsed (10 μM EBV-LCL of the donor and assayed on day 7 for tetramer\(^+\) CD8\(^+\) T cells. (B) PBMC stained with combinatorial encoded MiHA multimers, CD8 AlexaFluor 700, CD4-, CD14-, CD16- and CD19-FITC and Sytox Blue. Subsequently, cell populations were analyzed on the Cyan cytometer and re-analyzed by Kaluza 1.1. Cells were gated on CD8\(^+\)FITC ‘Sytox Blue’ lymphocytes, and the percentage of tetramer\(^+\) cells among CD8\(^+\) T cells is depicted.

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Concurrent Detection of MiHA-Specific CD8+ T Cells

A

APC

Qdot805
0.04%

Qdot855

Qdot705

B

PE

0.17%

APC

Qdot805

Qdot855

Qdot705
Multimer-positive cells. The percentage of multimer-
SMCY.A2 T cells after culturing with SMCY.A2-peptide pulsed donor EBV-LCL. Only the response observed in non-cultured PBMC show an increase of
SMCY.A2 set as described in Table 1. The combination of multimers coupled to PE and to Qdot605 shows a positive response, corresponding to multimer-
dot plots showing thawed PBMC of representative patient UPN348, stained with the combinatorial encoded MiHA-multimer assay using the peptide
doi:10.1371/journal.pone.0021266.g002
HLA-A2 monomers containing UV-predicted potential MiHA.

Figure 2. Detection of peptide-specific T cells by the combinatorial encoding MiHA-multimer assay. (A) Ten different flow cytometric
dot plots showing thawed PBMC of representative patient UPN348, stained with the combinatorial encoded MiHA-multimer assay using the peptide
set as described in Table 1. The combination of multimers coupled to PE and to Qdot605 shows a positive response, corresponding to multimer-
SMCY.A2 T cells. Cells were stained with MiHA multimers, CD8 AlexaFluor 700, CD4-, CD14-, CD16- and CD19-FITC and Sytox Blue. Subsequently, cell
collections were analyzed by flow cytometry. Cells were gated on CD8IFTIC “Sytox Blue” lymphocytes and corrected for single, triple or quadruple
multimer-positive cells. The percentage of multimer^CD8^ T cells is depicted. (B) Ten different dot plots showing PBMC of representative patient
UPN348 after culturing with SMCY.A2-peptide pulsed donor EBV-LCL. Only the response observed in non-cultured PBMC show an increase of
multimer-SMCY.A2 T cells. Staining and analysis was performed as described under (A).
doi:10.1371/journal.pone.0021266.g002

Figure 3. ELISA-based HLA-A2 affinity assay for 107
predicted potential MiHA. HLA-A2 monomers containing UV
degradable peptide were exchanged with 107 predicted MiHA peptides and HLA-A2 binding affinity was tested. Results show 32 low binders
(O.D<0.39), 33 intermediate binders (O.D 0.4–0.124) and 42 high binders
(O.D>1.25). Data are depicted as mean of triplicate wells.
doi:10.1371/journal.pone.0021266.g003

Materials and Methods

Patient material
All patients in this study were admitted in our transplantation
program from May 1996 onwards. Patients underwent HLA
identical allogeneic SCT using a T cell-depleted stem cell graft
containing a fixed number of 0.5×10^6 T cells/kg body weight. Several patients also underwent subsequent prophylactic or
therapeutic DLI. Peripheral blood samples of patients were
collected after written informed consent in ongoing clinical stem
cell transplantation protocols approved by the Radboud Universi-

Cell isolation and culture
CD8^ T cell bulk cultures were generated from peripheral blood
mononuclear cells (PBMC) obtained after SCT and cultured in
Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen,
Carlsbad, CA) supplemented with 10% human serum (HS;
Sanquin blood bank, Nijmegen, the Netherlands). After initial
stimulation, 100 IU/ml IL-2 (Chiron, Emeryville, CA) and 10 ng/
ml IL-15 (BD) were added at day 2. Flow cytometric analysis was
performed at day 7. All Epstein-Barr virus lymphoblastoid cell
lines (EBV-LCL) were cultured in IMDM/10% fetal calf serum
(FCS; Integro, Zaandam, The Netherlands).
SNP genotyping assays

Genotyping of HLA-matched SCT donor-recipient pairs for known MiHA was conducted using the KASPar assay, a fluorescence-based competitive allele-specific PCR that utilizes non-labeled primers (KBioscience, Herts, UK). Details of the process and primer sequence can be obtained from KBioscience.

SNP genotyping for verification of immunogenic epitopes was performed by Taqman SNP genotyping according to manufacturer’s recommendation (Applied Biosystems [ABI], Foster City, CA). Briefly, 10 ng of gDNA was amplified using ABI pre-designed SNP genotyping assays. 5 μl of gDNA (2 ng/μl) was combined with 12.5 μl of TaqMan Universal PCR Master Mix (2×) and 1.25 μl appropriate genotyping assay reagent containing specific forward and reverse primers and specific FAM/VIC probes (20×) in a total reaction volume of 25 μl. Reactions were run on the ABI 9700 thermal cycler in a 96 well format for 1 cycle at 95°C×10 minutes followed by 40 cycles at 92°C×15 seconds, 60°C×1 minute. A post-PCR plate read for FAM/VIC was performed on the ABI 9700HT and genotype calls were generated using SDS 2.3 software [ABI].

Figure 4. PBMC of a healthy donor enriched for virus-specific T cells. Peripheral blood of an HLA-A2+ healthy donor was enriched for EBV- and CMV-specific CD8+ T cells. Before culture there were low amounts of EBV- and CMV-specific CD8+ T cells present, which showed an increase after enrichment and 16 days of culture. Cells were stained with tetramers coupled to PE and gated on CD8 tetramer+. Analysis was performed on the Cyan cytometer.
doi:10.1371/journal.pone.0021266.g004
**Table 3. Percentage of dual-color tetramer−CD8+ T cells in PBMC samples of transplanted patients screened after MHC-multimer based enrichment and short-term expansion.**

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<tr>
<th>Gene</th>
<th>Candidate MiHA</th>
<th>UPN466</th>
<th>UPN541</th>
<th>UPN665</th>
<th>UPN407</th>
<th>UPN427</th>
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<td>WT-1</td>
<td>RMFPNAPYL</td>
<td>0.55a</td>
<td></td>
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<tr>
<td>BPI</td>
<td>KLOPYFQTL</td>
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<td>0.09</td>
<td>0.07</td>
<td>0.04</td>
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<tr>
<td>LAG3</td>
<td>LTGLQVSL</td>
<td></td>
<td>0.19b</td>
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<td></td>
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<tr>
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<td>0.10</td>
<td></td>
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<tr>
<td>GPR109b</td>
<td>FLEFLLLFL</td>
<td>0.23</td>
<td>0.04</td>
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*aKnown WT-1 epitope.

**Peptide-HLA-A2-based affinity ELISA**

Affinity of the predicted MiHA epitopes to HLA-A2 was tested by an HLA-A2 affinity streptavidin-based sandwich ELISA described by Rodenko et al. [9]. Briefly, 96-well plates were coated with streptavidin to which biotinylated monomers, containing all different predicted peptides, were added. Next, an HRP-conjugated anti-β2m antibody was added, binding to all stable MHC class I molecules. 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added and the green-colored oxidation product was measured at OD 414 nm.

**Enrichment of peptide-specific T cells**

PBMCs were obtained from CMV+ and EBV+ buffy coats (Sanquin blood bank, Nijmegen, the Netherlands) or from patients after SCT or DLI. All enrichments were performed on cryopreserved material. PBMC were stained with PE-labeled MHC multimers (1 μl of each individual PE-multimer for 10^7 PBMC) for 1 hour at 4°C. In case of the CMV/EBV enrichment a total of 2 μl PE-multimer for each specific epitope was added, while in case of enrichment for the candidate MiHA panel 79 μl PE-multimer mix consisting of 1 μl of each candidate and control MiHA epitope was added. Subsequently, cells were washed twice, and incubated with 20 μl magnetic beads coated with anti-PE antibody (Miltenyi Biotech, Bergisch Gladbach, Germany). Magnetically labeled cells were isolated by MACS (Miltenyi Biotech) using a MS Column following the manufacturer’s protocol. Eluted cells were washed and resuspended in IMDM supplemented with 10% human serum, 100 IU/ml IL-2 and 10 ng/ml IL-15 (BD). On day 1, 2×10^4 irradiated (60 Gy) autologous feeder cells were added along with 2,500 CD2/CD3/CD28 loaded anti-Biotin MACSiBead Particles (Miltenyi Biotech). On day 8, to prevent overgrowth by CD4+ T cells, T-cell cultures were depleted of residual CD4+ T cells via the addition of 50 μl of BD IMag anti-human CD4 particles (BD Biosciences) per 10^7 total cells and treatment per manufacturers’ protocol. Cultures were split and refreshed biweekly with medium plus cytokines. After 2–3 weeks, cell cultures were counted and tested for the presence of peptide-specific CD8+ T cells using combinatorial MHC multimer staining.

**Combinatorial MHC multimer staining**

Combinatorial MHC multimer stainings were performed on either thawed PBMC samples or ex vivo expanded T cell cultures. For this, 1×10^6 PBMCs or 1–5×10^5 cultured T cells were incubated with 1 μl of every single MHC multimer (final concentration 2 μg/ml per HMC based on initial monomer concentration) in a total volume of 80 μl for 15 min at room temperature in the dark. Subsequently, an antibody mixture consisting of 0.8 μl CD8-Alexa700 (Invitrogen, Carlsbad, CA), 4 μl CD4-FTTC (Beckman Coulter), 2 μl CD14-FTTC (BD), 2 μl CD19-FTTC (Dako), 6 μl CD16-FTTC (BD) was added, and cells were incubated for 30 min at 4°C. Finally, cells were washed twice and Sytox Blue (Invitrogen) was added at a final dilution of 1:5000 to allow dead cell exclusion. Data acquisition was performed on a Cyan-ADP analyzer (Beckman Coulter) and analyzed by Kaluza 1.1 software (Beckman Coulter). Antigen-specific T cells were quantified using an identical gating strategy as described by Hadrup et al. [7] and Velthuis et al [11]. First, viable Sytox Blue-negative, single-cell lymphocytes were gated. Subsequently, CD8-positive and FITC (CD4, CD14, CD16, CD19) negative cells were selected. MHC multimer positive cells were gated as CD8+ T cells positive in two MHC multimer channels (APC, PE, Qdot605, Qdot655, Qdot705 and Qdot800) and negative in the four other MHC multimer channels. In order to validate...
dual-color coded CD8+ T cells, PBMC were cultured in IMDM 10% HS and stimulated with irradiated EBV-LCL expressing the appropriate HLA restriction molecule. After initial stimulation, 100 IU/ml IL-2 (Chiron) and 10 ng/ml IL-15 (BD) was added at day 2. Primary T cell cultures were analyzed by flow cytometry at day 7.

Supporting Information

Table S1 Screening pool of high and intermediate binding HLA-A2 peptides set up with combinations of labeled MHC multimers.

(DOCX)
Acknowledgments

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References


