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Designing T-cells with desired T-cell receptor make-up for immunotherapy

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Kinetic preservation of dual-specificity of coprogrammed minor histocompatibility antigen-reactive virus-specific T-cells

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ABSTRACT

Adoptive transfer of antigen specific T-cells is an attractive strategy for the treatment of hematological malignancies. It has been demonstrated that T-cells recognizing minor histocompatibility antigens (mHags) selectively expressed on hematopoietic cells mediate anti-leukemic reactivity after allogeneic stem cell transplantation (allo-SCT). However, large numbers of T-cells with defined specificity are difficult to attain. An attractive strategy to obtain large numbers of leukemia-reactive T-cells is retroviral transfer of mHag-specific T-cell receptors (TCRs). TCR transfer into T-cells specific for persistent viruses may enable these T-cells to proliferate both after encountering viral antigens as well as mHags, increasing the possibility of in vivo survival. We analyzed whether the dual-specificity of the TCR transferred T-cells after repetitive stimulation via either the introduced anti-leukemic HA-2-TCR or the endogenous CMV-TCR was preserved. We demonstrate that after repetitive stimulation, T-cells skew to a population predominantly expressing the triggered TCR. However, HA-2-TCR transferred CMV-specific T-cells with high anti-leukemic HA-2-TCR expression but low CMV-TCR expression were able to persist and proliferate after repetitive stimulation with pp65. Moreover, HA-2-TCR transferred CMV-specific T-cells remained dual-specific after repetitive stimulation and TCR expression could be reverted after additional stimulation via the previously non stimulated TCR, restoring high avidity interactions. These data imply persistence of TCR transferred virus-specific T-cells with both anti-leukemic and anti-virus reactivity in vivo.

INTRODUCTION

Patients with relapsed hematological malignancies after HLA-matched allo-SCT can be succesfully treated with donor lymphocyte infusion (DLI)[1,2]. However, the beneficial graft versus leukemia (GVL) effect of donor lymphocytes is frequently accompanied by graft versus host disease (GVHD). GVL as well as GVHD appear to be caused by T-cells that are capable of recognizing mHags on patient cells[3-5]. mHags are immunogenic peptides derived from polymorphic proteins presented in the context of HLA molecules which are disparate between donor and recipient. T-cell responses against ubiquitously expressed mHags may be responsible for both GVL and GVHD. T-cells reactive with mHags selectively expressed on cells of the hematopoietic lineage may solely mediate GVL reactivity. HA-2 and HA-1 are examples of mHags selectively expressed in cells of the hematopoietic system and are presented in an HLA-class I-restricted fashion[6,7].

To separate the beneficial GVL from GVHD, adoptive transfer of T-cells recognizing mHags selectively expressed on cells of the hematopoietic system is a promising strategy. However, therapeutic cell numbers of mHag-specific T-cells are difficult to attain. An attractive alternative would be to equip T-cells with mHag-specific TCRs via retroviral gene transfer. Different studies have shown the effectiveness of TCR transferred T-cells in vitro[8-12] and in vivo[13-15]. Redirected T-cells were able to produce cytokines and exhibited Ag-specific cytolytic activity when triggered via the introduced TCR. Moreover, in mouse models it was shown that redirected T-cells could be activated via their introduced TCR, home to effector sites, and eradicate tumors. Recently, Rosenberg and colleagues demonstrated in a clinical trial the feasibility of adoptive transfer of TCR engineered T-cells in melanoma patients[14].

In most TCR gene transfer studies unselected peripheral blood T-cells were used as recipient T-cells. Transfer of TCRs into an unselected pool of T-cells may lead to transduction into regulatory T-cells capable of impairing the anti-leukemic immune reaction. Furthermore, in a pool of T-cells with a diverse TCR repertoire, a high number of different mixed TCR dimers with unknown specificity can be formed due to pairing of the retrovirally introduced TCR chains with the endogenously expressed TCR chains, increasing the probability of the formation of autoreactive mixed TCR dimers. Therefore, we previously proposed TCR transfer into virus-specific T-cells[16], since selection of these Ag-specific CD8+ T-cells leads to exclusion of regulatory T-cells. In addition, virus-specific memory T-cells generally consist of an oligoclonal population with restricted TCRαβ usage[17-21], minimizing the number of different mixed TCR dimers that can be formed. Furthermore, adoptive immunotherapy with EBV-specific T-cells in patients with post-transplant proliferative disease and CMV-specific T-cells as prophylaxis for CMV reactivation[22-24] in patients after SCT has proven to be a therapeutic strategy without toxicity or GVHD. Since EBV and CMV are examples of latent viruses, we hypothesize that due to frequent encounter with viral antigens and subsequent triggering of the endogenous TCR, TCR transferred virus-specific T-cells will survive for a prolonged period of time in vivo. Moreover, it was recently shown in a mouse model

that tolerization of one TCR could be overcome by signaling via the other TCR. In this model the function of the tolerized self-tumor reactive TCR of dual-T-cell receptor transgenic T-cells was rescued by proliferation induced via the virus-specific TCR, underlining the potency of TCR transfer into virus-specific T-cells[25].

We previously demonstrated that CMV-specific T-cells could be redirected into anti-leukemic T-cells by transfer of TCRs directed against the mHag HA-2 without the loss of their original specificity. T-cells were capable of exerting effector functions via their endogenous virus-specific TCR as well as via their introduced HA-2-specific TCR[16]. The TCR cell surface make up of HA-2-TCR transferred CMV-specific T-cells, however, varied. T-cells either highly expressed the endogenous TCR with a low expression of the introduced TCR, or highly expressed the introduced TCR with a low expression of the endogenous TCR, or expressed both TCRs intermediately at the cell surface. Different studies have shown that there is a threshold in expression of TCR complexes and costimulatory molecules needed for TCR signaling leading to proliferation[26,27]. Although both the HA-2-TCR and CMV-TCR used in this study are high-affinity TCRs, it is likely that differential TCR expression leads to differences in avidity and thus in proliferation. For long-term protection, we hypothesize that proliferative capacity via both TCRs will be important. When patients relapse, mHags will be abundantly present. However, when there is only minimal residual disease (MRD) it may be expected that HA-2-TCR transferred virus-specific T-cells will primarily encounter viral antigens latently present in the recipient, as the HA-2 antigen is only expressed by recipient hematopoietic cells. When frequent encounter of viral antigens would lead to selective survival of HA-2-TCR transferred CMV T-cells predominantly expressing the CMV-TCR incapable of proliferating via the HA-2-TCR, persistence in vivo of HA-2-TCR transferred CMV T-cells capable of controlling MRD may fall short.

In this study we analyzed the TCR expression, cytolytic potential and proliferation of HA-2-TCR transferred CMV-specific T-cells after repetitive stimulation with the CMV-pp65 antigen or the HA-2 antigen. We demonstrate that TCR-transferred virus-specific T-cells repetitively stimulated skewed to T-cells predominantly expressing one TCR. However, HA-2-TCR transferred CMV-specific T-cells with high anti-leukemic HA-2-TCR expression but low CMV-TCR expression were able to persist after repetitive stimulation with pp65. Moreover, HA-2-TCR transferred CMV-specific T-cells preserved their functional activity via both TCRs after repetitive stimulation, and TCR expression could be reverted after additional stimulation, restoring high avidity functionality of both the endogenous CMV-TCR and the introduced anti-leukemic HA-2-TCR.

MATERIALS AND METHODS

Construction of retroviral vectors and production of retroviral supernatant

The construction of retroviral vectors encoding for pp65 of HCMV AD169 and the TCR chains of the HA-2 reactive T-cell clone HA2.5 has been described previously[16]. Briefly, the

HA-2-TCR AV15 and HA-2-TCR BV18 chains were cloned into bicistronic retroviral vectors encoding the marker genes eGFP and ΔNGF-R[28], respectively. As control vectors, retroviral vectors were used containing eGFP or ΔNGF-R only.

HLA Class I tetrameric complexes and sorting by flow cytometry

Tetrameric HLA-A2 molecules in complex with CMV pp65 derived peptide NLVPMVATV (CMV tetramer) and the HA-2 derived peptide YIGEVLVSV (HA-2 tetramer) either PE- or APC-conjugated were constructed as previously described[29] with minor modifications. For flow cytometric analyses as well as FACS sorting, cells were labeled with tetramers for 1 hour at 4°C in RPMI without phenol, supplemented with 2% FBS, and washed two times or labeled with either anti-BV2 PE (Immunotech, Marseille, France), or anti ΔNGF-R either PE- (PharMingen, San Diego, California, USA) or APC-conjugated (Cedarlane Laboratories, Hornby, Ontario, Canada) for 30 minutes at 4°C.

Cells

For all stimulations and functional experiments EBV-transformed lymphoblastoid cell lines (EBV-LCLs) were used of an HLA-identical sibling pair with HA-2 disparity. EBV-LCL Z is HLA-A2 positive but HA-2 negative (EBV-Z), while EBV-LCL RZ is HLA-A2 and HA-2 positive (EBV-RZ HA-2). To obtain EBV-LCLs presenting endogenously processed pp65, EBV-Z was transduced with the lower matrix protein pp65 of CMV (EBV-Z pp65). EBV LCLs were maintained in IMDM supplemented with 10% FBS.

Malignant cells used in this study were chronic myeloid leukemia mononuclear cells (CML) CML-Z which is HLA A2 positive but HA-2 negative and CML-T which is HLA A2 and HA-2 positive. CML cells were thawed 1 day prior to testing and cultured in IMDM supplemented with 10% FBS.

Virus specific T-cells were isolated from peripheral blood of healthy individuals using CMV tetramers, as previously described[16], and expanded in T-cell medium containing IMDM supplemented with 5% FBS, 5% human serum and 100 IU/ml IL-2. T-cells were non specifically stimulated using 800 ng/ml PHA (Murex Biotec Limited, Dartford, UK) and irradiated autologous feeder cells. The CMV-specific T-cells were subsequently sorted using anti-BV2 PE, and non specifically restimulated, followed by retroviral transduction at day 2. For the transduction procedure recombinant human fibronectin fragments CH-296[30,31] were used. HA-2-TCR transduced BV2 positive T-cells were FACS sorted based on eGFP and NGF-R positivity, and the cells were expanded in bulk. T-cells were cultured in T-cell medium and either stimulated non specifically every 2 weeks with feeder cell mixtures containing $1x10^6$/ml irradiated allogeneic PBMCs (20Gy) and $1x10^5$/ml irradiated EBV-LCLs (50 Gy), or were repetitively stimulated with $1x10^6$/ml irradiated HLA-A2 negative allogeneic PBMCs and $1x10^5$/ml irradiated EBV-RZ HA-2 or EBV-Z pp65. Subsets of HA-2-TCR transduced virus-specific T-cells with various levels of TCR cell surface expression were sorted based on either high CMV-TCR and low HA-2-TCR expression using a combination of anti-BV2 mAb and HA-2 tetramer, or based on low CMV-TCR expression using only anti-BV2 mAb. No tetramers were used for

positive selection, since tetramer binding to the TCR can lead to specific stimulation[32]. Subsequently, sorted T-cells were tested functionally either directly after sorting, or after 7 days of stimulation with pp65 or HA-2. This study was approved by the Leiden University Medical Center institutional review board.

Cytotoxicity assay and PKH-26 based proliferation assay

Cytotoxicity assay was performed as previously described[16] using a standard 4 hours ^{51}Cr release assay at 10:1 effector-to-target ratios. The tests were done in duplicate. To test the capacity of T-cells to specifically proliferate in response to antigen, T-cells were labeled with PKH-26 (St. Louis, Missouri, USA) according to manufacturer's instructions, and stimulated with different feeder cell mixtures containing $1x10^6$/ml irradiated allogeneic HLA-A2 negative PBMCs in combination with $1x10^5$/ml target cells. The following targets were used: EBV-Z either unpulsed or pulsed with 1 µg/ml CMV-NLV or HA-2 YIG peptide, EBV-Z pp65 and EBV-RZ HA-2. Alternatively, T-cells were stimulated non-specifically by adding PHA. PKH dilution was analyzed at day 4 after stimulation using flow cytometry.

RESULTS

Skewing of TCR cell surface make-up upon specific TCR triggering

We hypothesize that for long-term protection the capacity of TCR transferred virus-specific T-cells to proliferate and exert effector functions in response to triggering via each TCR is important. Therefore, we studied whether repetitive Ag-specific stimulation of these dual-specific T-cells resulted in skewing of T-cells to a population predominantly expressing one TCR, incapable of exerting effector functions via the other TCR. For this purpose, T-cells recognizing the pp65 protein of CMV in the context of HLA-A2 (pp65-NLV) were isolated from PBMCs of healthy CMV seropositive individuals, transduced with the mHag-specific HA-2-TCR, and sorted on basis of marker gene expression. These TCR transferred virus-specific T-cells showed differences in TCR cell surface make up, which was stable for months after repetitive non-specific TCR triggering. The T-cells expressed either both TCRs at intermediate levels at the cell surface, or the endogenous TCR was highly expressed with a low expression of the introduced TCR, or the introduced TCR was highly expressed with a low expression of the endogenous TCR (Figure 1A). These HA-2-TCR transferred CMV T-cells exerted cytolytic activity directed against HA-2 expressing EBV-LCLs, as well as HA-2 expressing mononuclear CML cells (Figure 1B). To test whether all different T-cell subpopulations were able to persist after repetitive stimulation with either pp65 or HA-2, the dual-specific T-cells were stimulated with EBV-LCLs expressing either endogenously pp65 or HA-2. Differences in TCR expression were measured at day 7 after stimulation using CMV and HA-2 tetramers (Figure 1C). A gradual decrease in HA-2-TCR expression was observed after repetitive stimulation with pp65. Likewise, a gradual decrease in CMV-TCR expression was observed after repetitive stimulation with HA-2, while HA-2-TCR expression increased. To study whether changes in TCR expression could be

*Figure 1.: **(A)** CMV and HA-2 tetramer staining was analyzed for mock and HA-2-TCR transferred purified CMV T-cells previously sorted on eGFP en NGF-R marker gene positivity. Numbers indicate % T-cells per quadrant. **(B)** Mock CMV T-cells and HA-2-TCR transferred CMV T-cells were tested for anti-pp65 and anti-leukemic reactivity in an ^{51}Cr release assay. Target cells were EBV-Z, EBV-Z pp65, EBV-RZ HA-2, HLA-A2+ HA-2- CML-Z (CML-Z) and HLA-A2+ HA-2+ CML-T (CML-T). **(C)** CMV and HA-2 tetramer staining was analyzed for HA-2-TCR transferred CMV T-cells after every stimulation. T-cells were either stimulated three times with pp65 (black squares), or twice with pp65 and additionally with HA-2 (grey squares), or T-cells were stimulated three times with HA-2 (black circles) or twice with HA-2 and additionally with pp65 (grey circles). The percentage of tetramer positive T-cells is shown. **(D)** The dot plots of CMV and HA-2 tetramer staining of HA-2-TCR transferred CMV T-cells after three rounds of stimulation as indicated in **(C)** are depicted. Per quadrant % T-cells are indicated, numbers in brackets indicate MFI of the tetramer positive T-cell population. **(E)** HA-2-TCR transferred CMV T-cells stimulated three times with either pp65 or HA-2, and mock CMV T-cells were tested for cytotoxic activity against HA-2 and pp65 positive targets. The T-cells were tested at an E:T ratio of 10:1 against EBV-Z, pp65 peptide pulsed EBV-Z (EBV-Z + pp65 peptide), EBV-Z pp65, HA-2 peptide pulsed EBV-Z (EBV-Z + HA-2 peptide), and EBV-RZ HA-2. Data shown is representative for two independent experiments.*

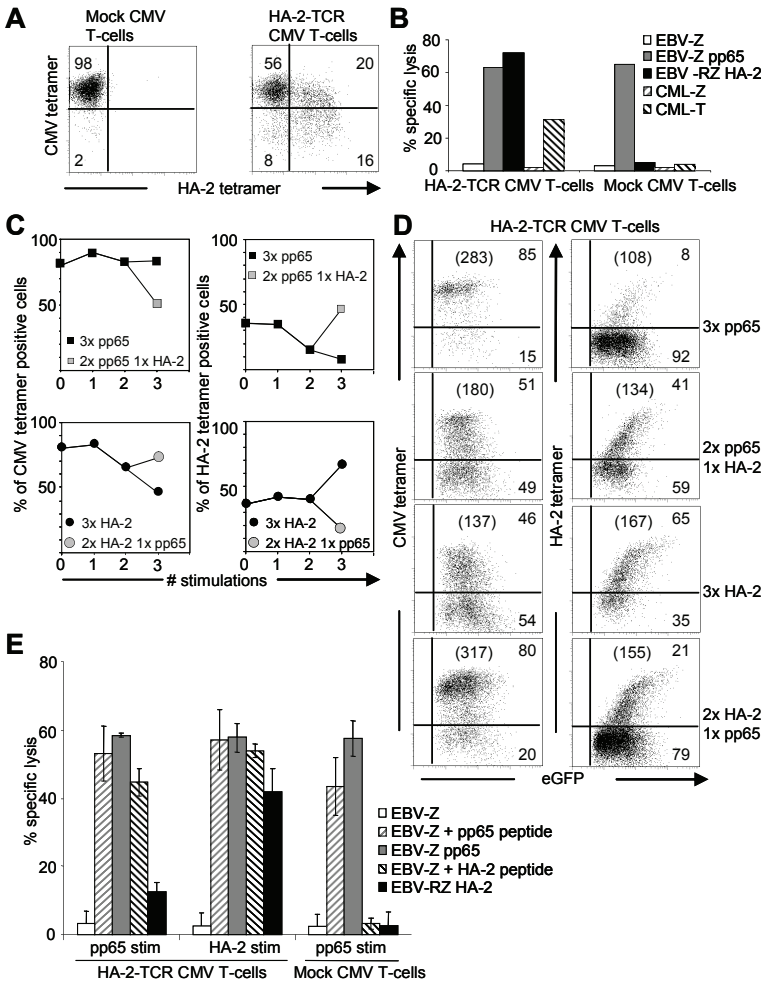

Figure 1. Cell surface expression and functional activity of the introduced and endogenous TCR after repetitive antigen-specific stimulation.

reversed by changing the stimulation, T-cells that were stimulated twice with pp65 were stimulated alternatively with HA-2. Likewise, T-cells that were stimulated twice with HA-2 were stimulated alternatively with pp65. The results demonstrate that by Ag-specific triggering of the previously non triggered TCR the TCR expression rapidly reverted (Figure 1C). On bases of the changed TCR make up (Figure 1D) we tested the HA-2 TCR transferred virus-specific T-cells stimulated repetitively with either only pp65 or only HA-2 for Ag-specific cytotoxic capacity (Figure 1E). Both T-cell populations were capable of killing HA-2 peptide loaded target cells, but the cytolytic activity of HA-2-TCR transduced T-cells repetitively stimulated with pp65 directed against target cells endogenously expressing HA-2 (EBV-RZ HA-2) was reduced. This was in accordance with the TCR expression, since in the T-cell population repetitively stimulated with pp65, only low numbers of HA-2 tetramer positive T-cells were present, while in the T-cell population repetitively stimulated with HA-2 still significant numbers of CMV tetramer positive T-cells were present.

These data illustrate that repetitive stimulation of HA-2-TCR transferred

CMV-specific T-cells with either HA-2 or CMV pp65 antigen resulted in preferential TCR expression of the triggered TCR, whereas expression of the non-triggered TCR gradually decreased. However, the T-cells with either predominant CMV or HA-2-TCR expression preserved their dual-specificity, although the level of reactivity in response to activation of the triggered TCR was higher than via the non-triggered TCR. In addition, changes in TCR expression could rapidly be reverted by Ag-specific triggering of the previously non-triggered TCR.

Generation and functionality of opposing T-cell subsets

To be able to dissect whether the difference in TCR make up after reverting the stimulation of T-cells predominantly expressing one TCR was due to selective outgrowth or due to differential TCR distribution, these T-cells were sorted into opposing subsets with either high CMV-TCR expression based on high CMV-TCR BV2 mAb staining and low HA-2 tetramer staining (Figure 2A; CMV-TCR^{hi}), or low CMV-TCR expression based on low CMV-TCR BV2 mAb staining (figure 2A; HA-2-TCR^{hi}). No tetramers were used for positive selection of the T-cells, since binding of the tetramers to the TCR would result in Ag-specific triggering via either the CMV or HA-2-TCR[32]. Directly after sorting, TCR expression of the sorted T-cell populations was analyzed using HA-2 tetramer and CMV-TCR BV2 mAb staining (Figure 2A). Both sorted T-cell subsets were positive for the marker genes eGFP and NGF-R (Figure 2B). To investigate whether T-cells almost exclusively expressing one TCR were still able to exert both HA-2 and pp65-specific cytolytic activity, the T-cells were tested in a

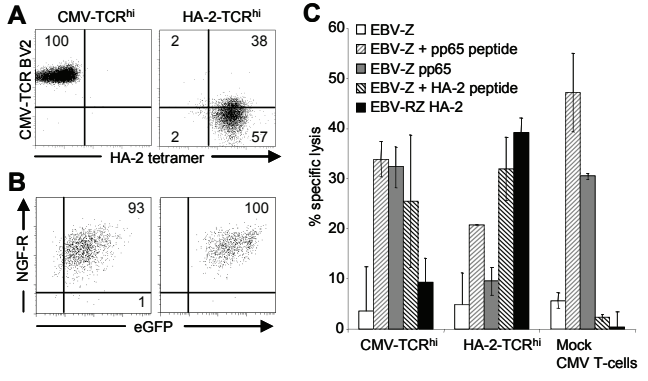

Figure 2. TCR cell surface expression and functional activity of opposing CMV-TCR^{hi} or HA-2-TCR^{hi} sorted T-cell populations.

cytotoxicity assay directly after sorting (Figure 2C). The CMV-TCR^{hi} T-cells exerted efficient cytotoxic activity against both pp65-peptide pulsed target cells and target cells endogenously expressing pp65, that was comparable to the mock transduced CMV T-cells. Although no HA-2-TCR expression could be measured on these T-cells using HA-2 tetramers (Figure 2A), the cells were still cytotoxic against HA-2 peptide pulsed target cells, but demonstrated marginal cytotoxic activity against target cells endogenously expressing HA-2, indicating that these T-cells only exhibit low avidity HA-2 reactivity (Figure 2C). The HA-2-TCR^{hi} T-cells efficiently lysed both HA-2 peptide pulsed target cells as well as the endogenous HA-2 positive target cells. Only low CMV-TCR expression could be measured on these T-cells, and the T-cells still demonstrated low cytotoxicity against pp65 peptide

*Figure 2: **(A)** HA-2-TCR transferred CMV T-cells were sorted on basis of CMV-TCR BV2^{high} and HA-2 tetramer^{low} (CMV-TCR^{hi}) or BV2^{low} staining (HA-2-TCR^{hi}), respectively. Directly after sorting, TCR expression of the sorted T-cell populations was analyzed using HA-2 tetramer and CMV-TCR BV2 mAb staining. **(B)** Both sorted T-cell subsets were analyzed for marker gene expression (eGFP and NGF-R). Numbers in **(A)** and **(B)** indicate % of cells per quadrant. **(C)** Mock CMV T-cells, CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell populations were tested for cytotoxic activity against HA-2 and pp65 positive target cells. One representative experiment out of four is shown.*

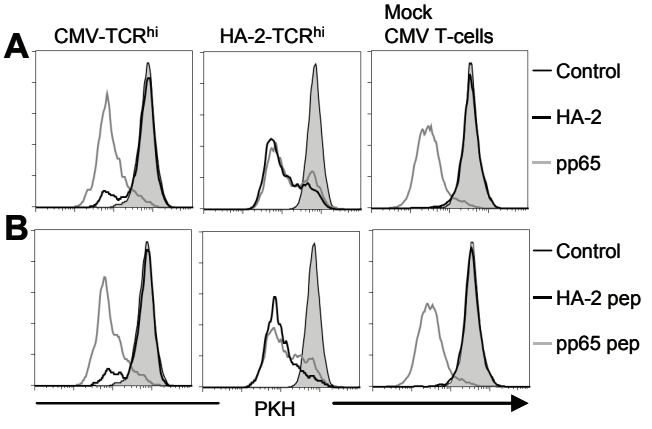

*Figure 3: **(A)** and **(B)** CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets and mock CMV T-cells were labeled with PKH-26, and stimulated Ag-specifically with HLA-A2 negative allogeneic feeders in combination with EBV-Z (Control), EBV-RZ HA-2 (A; HA-2) or HA-2 peptide pulsed EBV-Z (B; HA-2 pep) or EBV-Z pp65 (A: pp65) or pp65 peptide pulsed EBV-Z (B; pp65 pep). Only a small subset of CMV-TCR^{hi} sorted T-cells proliferate after HA-2-specific stimulation. One representative experiment out of four is shown.*

Figure 3. Proliferation of CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells after pp65 and HA-2-specific stimulation.

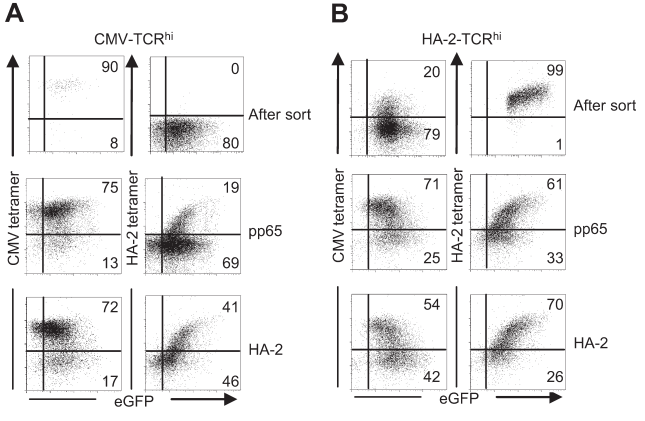

*Figure 4: CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets were stimulated pp65 or HA-2-specifically, and TCR expression was analyzed using CMV and HA-2 tetramers. **(A)** TCR expression of CMV-TCR^{hi} and **(B)** TCR expression of HA-2-TCR^{hi} sorted T-cells directly after sorting (after sort) or 7 days after stimulation with either EBV-Z pp65 (middle panel; pp65) or EBV-RZ HA-2 (lower panel; HA-2). Numbers indicate % of cells per quadrant. One representative experiment out of four is shown.*

Figure 4. TCR re-expression after restimulation of the CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets.

pulsed target cells, but only marginal cytotoxicity against target cells endogenously expressing pp65.

To study whether the sorted opposing T-cell subsets were able to proliferate upon HA-2 and pp65-specific stimulation, the T-cells were labeled with PKH-26 and analyzed using FACS at day 4. Both the CMV-TCR^{hi} and HA-2-TCR^{hi} T-cells were able to proliferate after stimulation with pp65 peptide pulsed EBV-LCLs or EBV-LCLs endogenously expressing pp65 (Figure 3A and B). Only a small percentage of the CMV-TCR^{hi} T-cells were capable of proliferating after stimulation with HA-2 peptide pulsed EBV-LCLS or EBV-LCLs endogenously expressing HA-2 (Figure 3A and B). The proliferation of HA-2-TCR^{hi} T-cells stimulated with HA-2 positive EBV-LCLs was similar to the proliferation induced by pp65 positive EBV-LCLs. Since the HA-2-TCR^{hi} T-cells were capable of proliferating both after HA-2 and pp65-specific stimulation, specific outgrowth as the main cause of reverting TCR make up was less plausible.

These results demonstrate that low CMV-TCR cell surface expression on HA-2-TCR^{hi} T-cells was sufficient for these cells to exert pp65-specific cytotoxic activity against pp65 peptide pulsed target cells, as well as pp65-specific proliferation. The low HA-2-TCR expression on CMV-TCR^{hi} T-cells was sufficient for these cells to exert specific cytotoxic activity against HA-2 peptide pulsed target cells, but was not enough for HA-2-specific proliferation. Therefore, it appears that the threshold of the endogenous TCR to induce proliferation and cytotoxic reactivity is more easily reached than the threshold of the introduced TCR, underlining the importance of targeting T-cells which will encounter antigens

that trigger their endogenous TCR in vivo to ensure persistence of TCR transferred T-cells.

Opposing T-cell subsets redistribute TCR expression on their cell surface after additional stimulation

To test whether also the CMV-TCR^{hi} and the HA-2-TCR^{hi} sorted T-cells were able to change their TCR make up after different specific stimulations, CMV-TCR^{hi} sorted T-cells (Figure 4A) and HA-2-TCR^{hi} sorted T-cells (Figure 4B) stimulated with EBV-LCLs presenting either endogenously processed pp65 or HA-2 were analyzed for TCR expression at day 7. After 7 days of Ag-specific stimulation CMV-TCR^{hi} sorted T-cells re-expressed the HA-2-TCR after stimulation with pp65 or HA-2 (Figure 4A). Although stimulation with HA-2 was not robust enough to induce proliferation of the CMV-TCR^{hi} sorted T-cells (Figure 3), it resulted in restored HA-2-TCR expression. Similarly, HA-2-TCR^{hi} sorted T-cells re-expressed the CMV-TCR after stimulation with pp65 or HA-2 (Figure 4B). These results demonstrate that also in the sorted T-cell subsets with predominant CMV or HA-2-TCR expression, T-cells are still capable of upregulating their TCR expression after stimulation via either the endogenous or introduced TCR.

Since we observed redistribution of TCR cell surface expression one week after stimulation (Figure 4), we investigated in a cytotoxicity assay whether cytolytic activity of CMV-TCR^{hi} or HA-2-TCR^{hi} sorted T-cells was improved (Figure 5A). After additional stimulation, only marginal differences in cytotoxic activity against HA-2 and pp65 positive target cells were observed between the CMV-TCR^{hi} and HA-2-TCR^{hi} T-cells (Figure 5A),

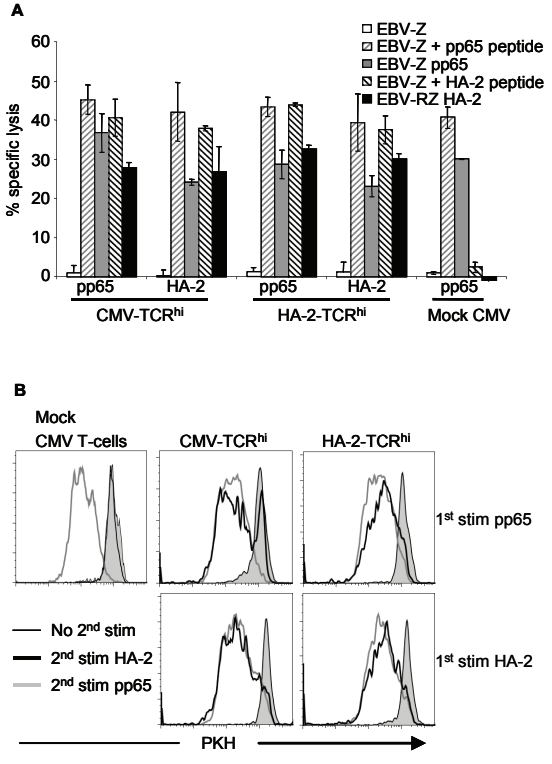

Figure 5. Comparable HA-2 and pp65 reactivity of CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells stimulated once after sorting.

corresponding with the restored co-expression of the HA-2- and CMV-TCR (Figure 4). All subsets displayed high cytotoxic activity against HA-2 positive and pp65 positive target cells, illustrating restored high avidity interactions.

*Figure 5: **(A)** CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cell subsets stimulated either pp65 or HA-2-specifically and mock CMV T-cells were analyzed for their pp65 and HA-2 reactivity 7 days after stimulation in a cytotoxicity assay. **(B)** Concurrently, the proliferative capacity of mock CMV T-cells, CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells was analyzed 7 days after their first specific stimulation. CMV-TCR^{hi} and HA-2-TCR^{hi} sorted T-cells stimulated directly after sorting with EBV-Z pp65 (upper panel; 1st stim pp65) or EBV-RZ HA-2 (lower panel; 1st stim HA-2) were either stimulated additionally with negative control EBV-Z (No 2nd stim; shadowed line), or with EBV-RZ HA-2 (2nd stim HA-2; black line), or with EBV-Z pp65 (2nd stim pp65; grey line). One representative experiment out of two is shown.*

To test whether the restored HA-2 and CMV-TCR co-expression also led to improved proliferation after stimulation via either endogenous or introduced TCR, the CMV-TCR^{hi} and the HA-2-TCR^{hi} T-cells were labeled with PKH-26, stimulated again with HA-2 or pp65 and analyzed using FACS at day 4 (Figure 5B). All CMV-TCR^{hi} T-cells and HA-2-TCR^{hi} T-cells stimulated with pp65 or HA-2 were able to proliferate vigorously after a second stimulation with pp65 positive EBV-LCLs. In contrast to the minimal amount of proliferation after HA-2-specific stimulation directly after sorting (Figure 3), most CMV-TCR^{hi} T-cells stimulated once with HA-2 were capable of proliferating after a second HA-2-specific stimulation. A small part of the CMV-TCR^{hi} T-cells stimulated once with pp65 was not able to proliferate upon HA-2-specific stimulation.

In conclusion, opposing T-cell populations are able to redistribute their TCRs at the cell surface after an additional Ag-specific stimulation, leading to restored functionality via both TCRs. These data imply that no loss of dual-specificity is likely to occur due to skewing of T-cells to a population predominantly expressing one TCR.

DISCUSSION

In this study, we demonstrate that TCR transferred virus-specific T-cells repetitively stimulated via one TCR remained dual reactive in response to triggering via both the endogenous and the introduced TCR. After repetitive stimulation of one TCR, TCR transferred T-cells preferentially expressed the triggered TCR, losing high avidity interaction via the previously non-triggered TCR. However, after a single stimulation via the previously non-triggered TCR, TCR expression reverted within one week. When the dual-specific T-cells were sorted in opposing CMV-TCR^{hi} T-cells and HA-2-TCR^{hi} T-cells, both subsets still demonstrated cytotoxic activity against HA-2 peptide pulsed target cells and CMV peptide pulsed target cells, respectively, but limited cytotoxic activity against targets presenting endogenously processed antigen, indicating loss of high avidity interactions. After additional stimulation, both subsets were able to re-express the HA-2 and CMV-TCR, respectively. When TCR expression was redistributed on the T-cells, high avidity functionality via both the endogenous and the introduced TCR was restored. Therefore, we speculate that also HA-2-TCR^{hi} T-cells are capable of persisting during MRD when HA-2-TCR transferred CMV T-cells will predominantly encounter viral antigens. Furthermore, we anticipate that HA-2-TCR transferred CMV T-cells after a long period of MRD are still able to gain anti-leukemic effector functions when the patient would relapse.

Directly after sorting T-cells predominantly expressed the CMV-TCR (CMV-TCR^{hi}) or the HA-2-TCR (HA-2-TCR^{hi}). However, after an additional stimulation TCR re-expression was observed. Surprisingly, HA-2-TCR re-expression was observed on CMV-TCR^{hi} TCR T-cells both after stimulation with HA-2 as well as with pp65, and CMV-TCR re-expression was observed on HA-2-TCR^{hi} T-cells both after stimulation with pp65 as well as with HA-2. TCR make up on transduced T-cells appears to be activation

dependent, however, a trend of preferential redistribution of the TCR being triggered was observed. It has been described that upon activation, T-cells enlarge and increase TCR expression[33-36] which is accompanied with restructuring compartmentalization of plasma membrane molecules[27,37,38]. Possibly, because of both increased TCR expression and localized high TCR density, HA-2-TCR on CMV-TCR^{hi} T-cells could be visualized using HA-2 tetramer staining after an additional stimulation, whereas this is not possible when HA-2-TCR is equally distributed along the cell membrane. Another possibility is that initial downregulation of the triggered TCR enabled surface expression of intracellular TCRs consisting of both the endogenous and introduced TCRs, whereas later on TCR expression will be dominated by newly synthesized previously triggered TCR. This would result in the preferential but not exclusive re-expression of the triggered TCR. It is evident that despite low CMV-TCR expression, HA-2-TCR^{hi} T-cells are capable of persisting during repetitive stimulation with pp65, although they do not proliferate as vigorously after stimulation with pp65 as CMV-TCR^{hi} T-cells. Even in a stringent selection of T-cells with predominant expression of either the introduced or the endogenous TCR, re-expression of the other TCR was observed, implying that TCR expression on these T-cells is dynamic rather than static.

Our results indicate that the threshold of the endogenous TCR to induce proliferation and cytotoxic reactivity is more easily reached than the threshold of the introduced TCR, underlining the importance of targeting T-cells which will encounter antigens that trigger their endogenous TCR in vivo to ensure persistence of TCR transferred T-cells. EBV and CMV are viruses which latently persist after initial infection and have to be continuously controlled by the immune system. Both in immunocompetent and immunocompromised hosts, immune responses result in viral containment in latent stage rather than virus eradication[39-41]. Therefore, we propose to use EBV or CMV-specific T-cells as host cells for TCR transfer. When there is only minimal residual disease it may be expected that HA-2-TCR transferred virus-specific T-cells will primarily encounter viral antigens latently present in the recipient, as the HA-2 antigen is only expressed by recipient hematopoietic cells. We hypothesize that low dose triggering of the endogenous TCR due to the persistence of the virus will also boost the anti-leukemic immune response mediated via the HA-2-TCR.

To ensure persistence and correct homing of transduced virus-specific T-cells it is discussed that different memory subsets should be used[42,43], or virus-specific T-cells responsible for the immunodominant response in the donor should be selected[21,44]. Recent studies demonstrated that distinct memory subsets are raised in different viral infections[45,46]. Even within one virus-specific memory response distinct subsets of virus-specific CD8+ T-cells can be found. For example, the CD8+ memory T-cells specific for EBV lytic antigens predominantly have a more differentiated effector memory phenotype, whereas CD8+ memory T-cells specific for EBV latent antigens predominantly have a central memory phenotype[40]. Therefore it is hypothesized that phenotype of CD8+ memory T-cells could well be dictated by different routes of antigen exposure. Based

on phenotypic characteristics CD8+ memory T-cells specific for CMV are mainly effector-type or late memory T-cells[41]. However, studies have demonstrated that CD8+ memory T-cells specific for CMV are able to respond with renewed clonal expansion upon viral reactivation[23,47], suggesting that phenotypic classification alone is not indicative for functional characteristics. We therefore would like to use for clinical application a pool of CMV or EBV-specific T-cells with distinct phenotypic characteristics, resulting in virus-specific T-cells with different functional characteristics and homing capacities.

In conclusion, although after repetitive stimulation HA-2-TCR transferred CMV-specific T-cells skew to populations predominantly expressing one TCR, all subsets are able to persist and repopulate after stimulation via the previously non-triggered TCR. Therefore we conclude that TCR transduced virus-specific T-cells behave favorably in view of future clinical applications.

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