

# Extracellular Domains of CD8 $\alpha$ and CD8 $\beta$ Subunits Are Sufficient for HLA Class I Restricted Helper Functions of TCR-Engineered CD4<sup>+</sup> T Cells

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## Abstract

By gene transfer of HLA-class I restricted T-cell receptors (TCRs) (HLA-I-TCR) into CD8<sup>+</sup> as well as CD4<sup>+</sup> T-cells, both effector T-cells as well as helper T-cells can be generated. Since most HLA-I-TCRs function best in the presence of the CD8 co-receptor, the CD8 $\alpha\beta$  molecule has to be co-transferred into the CD4<sup>+</sup> T-cells to engineer optimal helper T-cells. In this study, we set out to determine the minimal part of CD8 $\alpha\beta$  needed for optimal co-receptor function in HLA-I-TCR transduced CD4<sup>+</sup> T-cells. For this purpose, we transduced human peripheral blood derived CD4<sup>+</sup> T-cells with several HLA-class I restricted TCRs either with or without co-transfer of different CD8 subunits. We demonstrate that the co-transduced CD8 $\alpha\beta$  co-receptor in HLA-I-TCR transduced CD4<sup>+</sup> T-cells behaves as an adhesion molecule, since for optimal antigen-specific HLA class I restricted CD4<sup>+</sup> T-cell reactivity the extracellular domains of the CD8 $\alpha$  and  $\beta$  subunits are sufficient.

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## Introduction

Adoptive transfer of T-cells is a strategy used to target both solid tumors[1] and leukemia[2–4]. By introducing well-characterised TCRs via gene transfer large numbers of T-cells with defined antigen-specificity can be obtained without long in vitro culture periods. Transfer of HLA-I-TCRs into CD8<sup>+</sup> T-cells demonstrated redirected antigen-specificity[5–10] and recently the in vivo efficacy of adoptively transferred TCR transduced (td) T-cells was demonstrated in melanoma and synovial cell sarcoma patients[11–13]. For optimal maintenance of functional CD8<sup>+</sup> immune responses in vivo, however, antigen-specific CD4<sup>+</sup> T cells may play an essential role[14,15]. By TCR engineering of CD8<sup>+</sup> as well as CD4<sup>+</sup> T-cells, both effector T-cells as well as helper T-cells with the same specificity can be generated. However, since most HLA-I-TCRs function best in the presence of the CD8 co-receptor, the CD8 molecule has to be co-transferred into the CD4<sup>+</sup> T-cells to engineer optimal helper T-cells[16,17].

The CD8 molecule can be expressed as an  $\alpha\alpha$  or an  $\alpha\beta$  dimer, but is on peripheral TCR $\alpha\beta$  T-cells mostly expressed as an  $\alpha\beta$  dimer[18–23]. The  $\alpha$  subunit of CD8 binds to the non-polymorphic residues of the  $\alpha 3$  domain of HLA class I, thereby enhancing the avidity of the TCR/MHC complex[24]. The cytoplasmic tail of the  $\alpha$  subunit directly associates with the protein tyrosine kinase Lck[25–28], promoting signal transduction after T-cell activation. The intracellular domain of the  $\beta$  subunit enhances the association of CD8 $\alpha$  with lipid raft localized Lck[29,30] and the linker for activation of T-cells (LAT)[31,32]. Although the mechanism is not clear yet, it has been demonstrated

that CD8 $\alpha\beta$  heterodimers bind MHC class I molecules more avidly than CD8 $\alpha\alpha$  homodimers[32–34].

Previously, it was reported that for optimal proliferation, cytokine production and cytotoxicity of HLA-I-TCR td CD4<sup>+</sup> T-cells co-expression of CD8 $\alpha\beta$  was needed whereas co-expression of CD8 $\alpha\alpha$  only marginally increased functional activity[16,17]. Here, we studied whether the extracellular and/or intracellular part of CD8 $\alpha$  and CD8 $\beta$  were required for this increased functional activity.

## Results and Discussion

### Extracellular CD8 $\alpha$ and $\beta$ are required and sufficient to elicit HLA class I restricted IFN- $\gamma$ production

To verify that functional activity of high-affinity HLA-I-TCR transduced (td) CD4<sup>+</sup> T-cells was improved by the transfer of CD8 $\alpha\alpha$  or CD8 $\alpha\beta$  co-receptor, CMV-specific CD4<sup>+</sup> T-cells were transduced with the high-affinity HA-2-TCR with either only CD8 $\alpha$  or with both the CD8 $\alpha$  and CD8 $\beta$  subunits and purified based on CD8 $\alpha\alpha$  or CD8 $\alpha\beta$  expression. T-cells were tested against LCLs pulsed with pp65 peptide stimulating the endogenous CMV-TCR, or with either HA-2 peptide or HA-2<sup>+</sup> LCLs stimulating the introduced HA-2-TCR, and antigen-specific IFN- $\gamma$  production was measured (Figure 1A). As can be observed, HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells co-transferred with CD8 $\alpha\alpha$ , CD8 $\alpha\beta$  or negative for CD8 were equally potent in recognizing pp65 peptide pulsed target cells. In addition, all three populations were able to recognize HA-2 peptide loaded target cells. However, only the CD8 $\alpha\beta$  co-transferred T-cells were able to recognize

endogenously processed and presented HA-2 (Figure 1A). These results confirmed previous studies demonstrating that retroviral introduction of CD8 $\alpha\beta$  increased the functional activity mediated via the introduced TCR of the TCR td CD4<sup>+</sup> T-cells [16,17].

To more precisely determine the part of the CD8 $\alpha\beta$  coreceptor responsible for increased functional activity of the HLA-I-TCR td CD4<sup>+</sup> T-cells, we constructed intracellularly truncated CD8 $\alpha$  ( $\Delta$ CD8 $\alpha$ ), Lck mutated CD8 $\alpha$  (CD8 $\alpha$  Lck), and intracellularly truncated CD8 $\beta$  ( $\Delta$ CD8 $\beta$ ). The HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells were transduced with the different CD8 $\alpha$  and CD8 $\beta$  constructs, purified based on CD8 $\alpha\alpha$  or CD8 $\alpha\beta$  expression, and used as effector T-cells in the experiments described here above (Figure 1B). Results were similar to the experiments with unmodified (wt)CD8 $\alpha$  and wtCD8 $\beta$  co-transferred HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells (Figure 1A). Only TCR td CD4<sup>+</sup> T-cells co-transferred with both CD8 $\alpha$  and CD8 $\beta$  produced significant amounts of IFN- $\gamma$  after stimulation with HA-2<sup>+</sup> LCLs, irrespective of whether the co-transferred CD8 $\alpha$  and  $\beta$  subunits were intracellularly truncated or whether the Lck binding motif of the CD8 $\alpha$  subunit was mutated (Figure 1B). Mock td CMV-specific CD4<sup>+</sup> T cells specifically produced IFN- $\gamma$  only after stimulation with pp65 peptide pulsed LCLs (data not shown). These results demonstrate that for optimal HLA class I restricted IFN- $\gamma$  production of the TCR td CD4<sup>+</sup> T-cells, co-transfer of the extracellular domains of CD8 $\alpha$  and  $\beta$  is required but that the intracellular domains can be dismissed.

Polyfunctionality of CD4<sup>+</sup> T cells is important for optimal helper function. Therefore, we studied the capacity of HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells co-transferred with different CD8 constructs to produce not only IFN- $\gamma$ , but also TNF- $\alpha$  and IL-2 and upregulate CD40L (Figure 1C). TCR td CD4<sup>+</sup> T-cells co-transferred with either wtCD8 $\alpha\beta$  or  $\Delta$ CD8 $\alpha\beta$  produced significantly more cytokines and demonstrated significantly more CD40L upregulation after stimulation with either HA-2 pulsed or HA-2<sup>+</sup> LCLs ( $p < 0.05$  indicated with asterisks) than CD8 negative or CD8 $\alpha\alpha$  expressing TCR td CD4<sup>+</sup> T-cells. No significant difference in cytokine production or CD40L upregulation after HA-2 specific stimulation was observed between TCR td CD4<sup>+</sup> T-cells co-transferred either with wtCD8 $\alpha\beta$  or  $\Delta$ CD8 $\alpha\beta$ . The results in Figure 1C demonstrate that for IFN- $\gamma$ , TNF- $\alpha$  and IL-2 production as well as for CD40L upregulation after stimulation with HA-2<sup>+</sup> LCLs co-transfer of CD8 $\alpha$  and  $\beta$ , and most importantly the extracellular domains of these CD8 subunits, is required.

In conclusion, to generate polyfunctional HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells, co-transfer of both CD8 $\alpha$  and  $\beta$  is required, but the intracellular domains of these CD8 subunits can be dismissed.

### $\Delta$ CD8 $\alpha$ and $\Delta$ CD8 $\beta$ improve HLA-class I restricted avidity similarly efficient as wtCD8 $\alpha$ and wtCD8 $\beta$

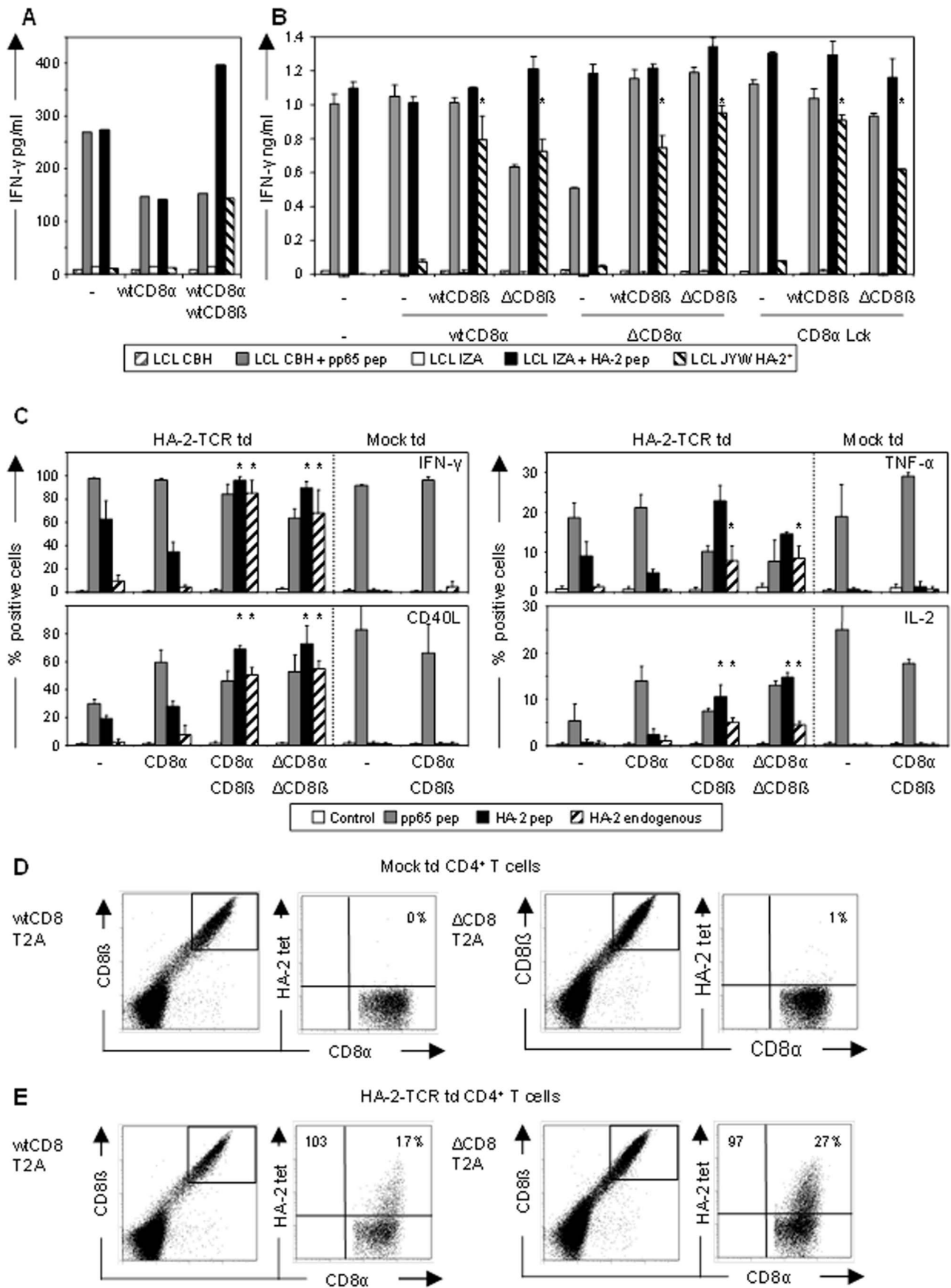
To analyze whether TCR td CD4<sup>+</sup> T-cells co-transferred with the different CD8 subunits bind with similar affinity the HLA-peptide complex, HA-2-tetramer staining was analyzed for both mock and HA-2-TCR td CD4<sup>+</sup> T-cells (Figure 1D and E). No specific HA-2 tetramer staining could be observed for mock and TCR td CD4<sup>+</sup> T-cells without CD8 $\alpha\beta$  co-transfer (data not shown). However, co-transfer of CD8 $\alpha$  alone or transfer of CD8 $\alpha\beta$  using two separate retroviral vectors resulted in aspecific staining of every tetramer added (data not shown). Therefore, mock and HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells were co-transferred with multicistronic vectors in which the CD8 $\alpha$  and CD8 $\beta$  molecules were linked with a 2A sequence resulting in equimolar levels of both CD8 $\alpha$  and  $\beta$  molecules (Figure 1D and E), and

analyzed for tetramer staining. As can be seen in Figure 1D, no aspecific staining of HA-2 tetramer is detected on mock transduced CD4<sup>+</sup> T-cells co-transferred with the multicistronic CD8 $\alpha\beta$  vectors, whereas TCR td CD4<sup>+</sup> T-cells co-transferred with the wtCD8 $\alpha\beta$  or  $\Delta$ CD8 $\alpha\beta$  multicistronic vectors demonstrated identical HA-2 tetramer staining, indicating similar avidity for HLA-A2/HA-2-peptide complex (Figure 1E).

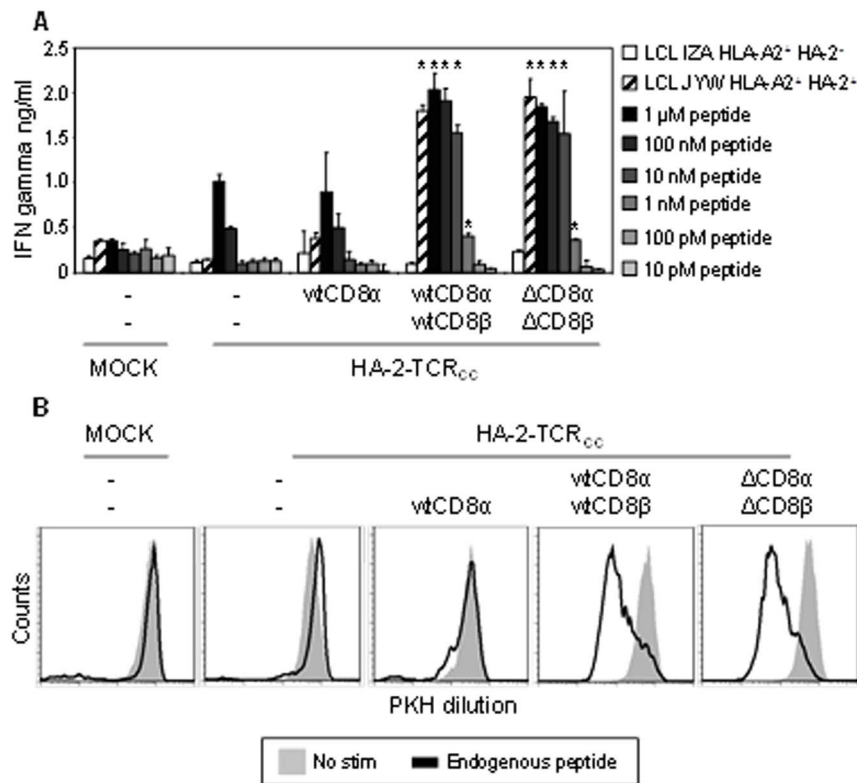
Next, we studied whether co-transfer of wtCD8 $\alpha\beta$  or  $\Delta$ CD8 $\alpha\beta$  equally improved the function of CD4<sup>+</sup> T-cells transduced with a next generation HA-2-TCR<sub>CC</sub>, that was codon optimized and cysteine modified to improve TCR cell surface expression. For this purpose, CMV-specific CD4<sup>+</sup> T-cells were transduced with the next generation HA-2-TCR<sub>CC</sub> either without or in combination with different CD8 subunits, purified using flow cytometry based cell sorting and tested for HA-2-specific IFN- $\gamma$  production against target cells loaded with titrated concentrations of the HA-2 peptide as well as against target cells that endogenously process and present the antigen (HA2<sup>+</sup> target cells). As demonstrated in Figure 2A, the HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells expressing either wtCD8 $\alpha\beta$  or  $\Delta$ CD8 $\alpha\beta$  were equally reactive against HA-2 peptide loaded target cells, and approximately a 100 fold more sensitive compared to CD8 negative or CD8 $\alpha\alpha$  expressing HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells. In addition, whereas CD8 negative or CD8 $\alpha\alpha$  expressing HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells demonstrated no or low reactivity against HA-2<sup>+</sup> target cells, the  $\Delta$ CD8 $\alpha\beta$  and wtCD8 $\alpha\beta$  expressing HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells were highly reactive against HA2<sup>+</sup> target cells. To confirm the significantly increased sensitivity of the wtCD8 $\alpha\beta$  and  $\Delta$ CD8 $\alpha\beta$  expressing HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells compared to the CD8 negative or CD8 $\alpha\alpha$  expressing cells, we performed a proliferation assay in which we stimulated PKH-labeled T-cells with HA-2<sup>+</sup> and HA-2<sup>-</sup> target cells and analyzed proliferation by measuring PKH dilution at day 5 after stimulation. As can be observed in Figure 2B, wtCD8 $\alpha\beta$  and  $\Delta$ CD8 $\alpha\beta$  HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells proliferated equally efficient after stimulation with HA-2<sup>+</sup> target cells, whereas for the CD8 negative or CD8 $\alpha\alpha$  expressing T-cells no antigen-specific proliferation was observed. These data indicate that co-transfer of the extracellular parts of CD8 increase avidity of HA-2-TCR expressing CD4<sup>+</sup> T-cells for HLA-A2<sup>+</sup> HA-2<sup>+</sup> target cells approximately a 100-fold. This increase in avidity is necessary to elicit efficient IFN- $\gamma$  production and proliferation of HA-2-TCR and HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells after stimulation with physiologically relevant levels of HA-2.

### In general, HLA-I-TCR td CD4<sup>+</sup> T-cells require co-transfer of only the extracellular CD8 $\alpha\beta$ domains

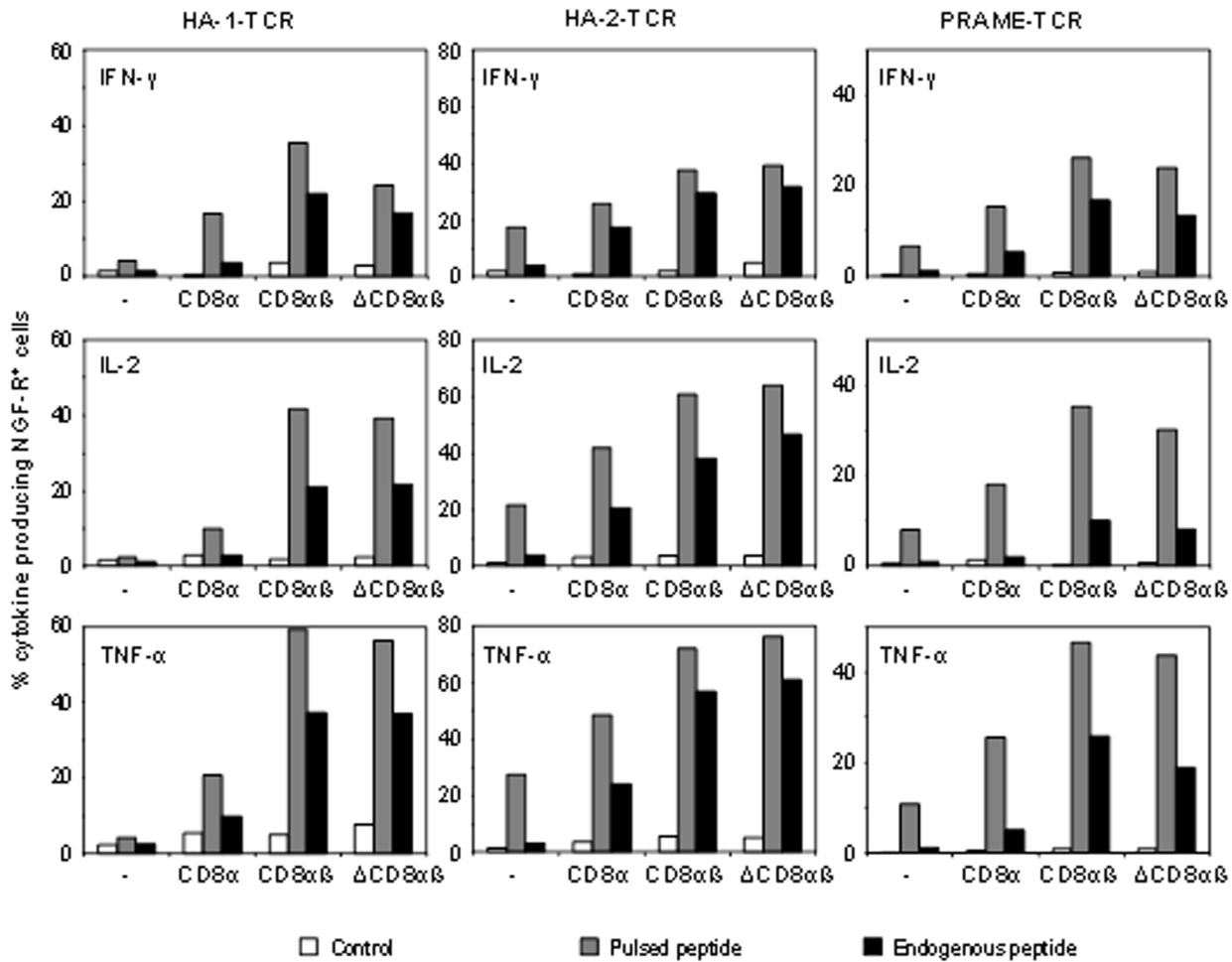
To confirm the generality of these data, polyclonal peripheral CD4<sup>+</sup> T-cells were transduced with next generation high-affinity TCRs specific for HA-1, HA-2 or PRAME and were co-transferred with the different CD8 constructs. Results presented in Figure 3 demonstrate that CD4<sup>+</sup> T-cells transduced with either HA-1-TCR<sub>CC</sub>, HA-2-TCR<sub>CC</sub> or PRAME-TCR<sub>CC</sub> displayed IFN- $\gamma$ , IL-2- and TNF- $\alpha$  production only after stimulation with peptide pulsed target cells (1  $\mu$ g/ml), and not after stimulation with antigen-positive target cells expressing endogenously processed antigen. Introduction of only the wtCD8 $\alpha$  molecule induced some cytokine production against peptide pulsed target cells and antigen-positive target cells. However, when wtCD8 $\alpha\beta$  or  $\Delta$ CD8 $\alpha\beta$  were introduced, substantial percentages of transduced T-cells produced IFN- $\gamma$ , IL-2 and TNF- $\alpha$  both after stimulation with peptide pulsed target cells or antigen-positive target cells. These results demonstrate a general trend in requirement and sufficiency of co-transfer of the extracellular domains of CD8 $\alpha$  and  $\beta$  for HLA-class I restricted helper functions.



**Figure 1. HLA-I-TCR td CD4<sup>+</sup> T-cells co-transferred with wtCD8 $\alpha\beta$  or intracellularly modified CD8 $\alpha\beta$  demonstrate equal effector functions.** To study the minimal part of CD8 needed for optimal co-receptor function in HLA-I-TCR td CD4<sup>+</sup> T-cells, HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells (A) co-transferred with wtCD8 $\alpha\alpha$  or wtCD8 $\alpha\beta$  co-receptor, or (B) co-transferred with either wtCD8 $\alpha$ ,  $\Delta$ CD8 $\alpha$  or CD8 $\alpha$  Lck in combination with either wtCD8 $\beta$  or  $\Delta$ CD8 $\beta$  were purified and used in a stimulation assay. Td T-cell populations were tested against HLA-DR1<sup>+</sup> LCL-CBH either unpulsed (grey striped bars) or pulsed with pp65 peptide (grey bars), or against HLA-A2<sup>+</sup> HA-2<sup>-</sup> LCL-IZA either unpulsed (white bars) or pulsed with HA-2 peptide (black bars), or against HLA-A2<sup>+</sup> HA-2<sup>+</sup> LCL-JYW (black striped bars). IFN- $\gamma$  production was measured after 18 h of stimulation in duplicate, and a representative experiment out of 3 is depicted. The IFN- $\gamma$  production of the different CD8 $\alpha\beta$  expressing TCR td T-cells was compared to the IFN- $\gamma$  production of CD8 $\alpha\alpha$  expressing TCR td T-cells within their group using students' t-test. P-values <0.05 are indicated with an asterisk. (C) To study whether co-transfer of CD8 would also result in polyfunctional helper functions of TCR td CMV-specific CD4<sup>+</sup> T-cells, both mock and HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells with or without co-transfer of different CD8 subunits as indicated in the figure were stimulated with HLA-DR1<sup>+</sup> LCL-CBH pulsed with pp65 peptide (grey bars; pp65 pep), unpulsed HLA-A2<sup>+</sup> HA-2<sup>-</sup> LCL-IZA (white bars; control), HA-2 peptide pulsed HLA-A2<sup>+</sup> HA-2<sup>-</sup> LCL-IZA (black bars; HA-2 pep) or HLA-A2<sup>+</sup> HA-2<sup>+</sup> LCL-JYW (striped bars; HA-2 endogenous). After 5 h of stimulation, T-cells were stained with anti-IFN- $\gamma$ , anti-TNF- $\alpha$ , anti-CD40L and anti-IL-2 mAbs and were analyzed using flow cytometry. The percentage of IFN- $\gamma$ , TNF- $\alpha$  and IL-2 producing or CD40L expressing T-cells after stimulation is depicted. The percentages of cytokine producing and CD40L upregulating CD8 $\alpha\beta$  expressing TCR td T-cells that were significantly higher than CD8 negative and CD8 $\alpha\alpha$  expressing TCR td T-cells (p-values <0.05) are indicated with an asterisk. (D/E) To study differences in avidity between HLA-I-TCR td CD4<sup>+</sup> T-cells co-transferred with the different CD8 $\alpha$  and CD8 $\beta$  constructs, HA-2 tetramer staining was analyzed. (D) Mock or (E) HA-2-TCR td CD4<sup>+</sup> T-cells co-transferred with either wtCD8 $\alpha$ -T2A-wtCD8 $\beta$  (wtCD8 T2A; left dot plots) or  $\Delta$ CD8 $\alpha$ -T2A- $\Delta$ CD8 $\beta$  ( $\Delta$ CD8 T2A, right dot plots) were stained with anti-CD8 $\alpha$  and  $\beta$  mAbs and HA-2-tetramers and analyzed using flow cytometry. Populations were gated on CD8 $\alpha\beta$  positive expression and HA-2 tetramer staining is depicted for the gated populations. Percentages of HA-2-tetramer positive T-cells are indicated in the upper right and MFI of the HA-2-tetramer staining in the upper left of the dot plots. Data shown are representative for 2 independent experiments.  
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**Figure 2. Improved HLA-class I restricted avidity of CD8 $\alpha\beta$  expressing HA-2-TCR td CD4<sup>+</sup> T-cells results in improved proliferation.** (A) To study whether co-transfer of CD8 would also improve the peptide sensitivity of CD4<sup>+</sup> T-cells transduced with a next generation HA-2-TCR, both mock and HA-2-TCR td CMV-specific CD4<sup>+</sup> T-cells with or without co-transfer of different CD8 subunits as indicated in the figure were purified using flow cytometry based cell sorting and stimulated with unpulsed HLA-A2<sup>+</sup> HA-2<sup>-</sup> LCL IZA (white bars; LCL IZA), HLA-A2<sup>+</sup> HA-2<sup>-</sup> LCL-IZA pulsed with decreasing concentrations of HA-2 peptide (range 1  $\mu$ M-10 pM) or HLA-A2<sup>+</sup> HA-2<sup>+</sup> LCL JYW (striped bars; LCL JYW). IFN- $\gamma$  production was measured after 18 h of stimulation in duplicate, and a representative experiment out of 2 is depicted. The IFN- $\gamma$  production of  $\Delta$ CD8 $\alpha\beta$  and wtCD8 $\alpha\beta$  expressing HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells significantly higher (p-values <0.05) than CD8 negative or CD8 $\alpha\alpha$  expressing HA-2-TCR<sub>CC</sub> td CD4<sup>+</sup> T-cells is indicated with an asterisk. (B) To investigate their proliferative capacity, both mock and HA-2-TCR td CD4<sup>+</sup> T-cells without CD8 or co-transferred with wtCD8 $\alpha$ , wtCD8 $\alpha\beta$ , or  $\Delta$ CD8 $\alpha\beta$  were purified based on markergene expression and CD8 cell surface expression and were either not stimulated (filled histograms) or stimulated with HLA-A2<sup>+</sup> HA-2<sup>+</sup> LCL-JYW (thick black line). Histograms depict PKH dilution measured 5 days after stimulation, and a representative example of 2 independent experiments is depicted.  
doi:10.1371/journal.pone.0065212.g002



**Figure 3. In general, co-transfer of the extracellular domains of CD8 $\alpha$  and  $\beta$  is required and sufficient.** To confirm the generality of the previous data, total CD4<sup>+</sup> T-cells were transduced with codon optimized and cysteine modified HA-1-, HA-2- or PRAME-TCR (transduction efficiency 48%, 48% and 22%, respectively) either with or without co-transfer of different CD8 molecules, as indicated in the figure. One week after transduction, non-purified TCR td CD4<sup>+</sup> T-cells were stimulated and tested for cytokine production using flow cytometry. HA-1- or HA-2-TCR td CD4<sup>+</sup> T-cells were stimulated either with HA-1 or HA-2 peptide pulsed or unpulsed HLA-A2<sup>+</sup> HA-1<sup>-</sup> HA-2<sup>-</sup> LCL-IZA, or HLA-A2<sup>+</sup> HA-1<sup>+</sup> HA-2<sup>+</sup> LCL-MRJ, and PRAME-TCR td CD4<sup>+</sup> T-cells were stimulated either with PRAME peptide pulsed or unpulsed HLA-A2<sup>+</sup> PRAME<sup>-</sup> melanoma cells, or HLA-A2<sup>+</sup> PRAME<sup>+</sup> melanoma cells. 5 h After stimulation, T-cells were permeabilized and stained with anti-NGF-R in combination with either anti-IFN- $\gamma$  (upper panel), anti-IL-2 (middle panel) or anti-TNF- $\alpha$  (lower panel), and analyzed using flow cytometry. The percentage of markergene positive and CD8 positive T-cells producing cytokines after stimulation with antigen-negative cells (white bars; control), peptide pulsed cells (grey bars; pulsed peptide) or antigen-positive cells (black bars; endogenous peptide) is indicated. doi:10.1371/journal.pone.0065212.g003

**Conclusions**

We hypothesize that CD8 co-transfer in antigen-experienced CD4<sup>+</sup> T-cells potentially poses the risk of overstimulation. To minimize the risk of overstimulation in HLA class I restricted TCR transduced CD4<sup>+</sup> T-cells, we analyzed in this study whether co-transfer of a signaling deficient CD8-co-receptor would also result in optimal HLA class I restricted functionality.

We confirmed that for optimal helper functions of HLA-I-TCR td CD4<sup>+</sup> T-cells co-expression of CD8 $\alpha\beta$  co-receptors is superior to CD8 $\alpha\alpha$  co-receptors, and CD8 $\alpha\beta$  co-expressing T-cells were superior in producing IFN- $\gamma$ , TNF- $\alpha$ , and IL-2, in upregulating CD40L, and in antigen specific proliferation. Expression of the extracellular domains of CD8 $\alpha\beta$  was required both for CD4<sup>+</sup> T-cells transduced with unmodified HA-2-TCR, as well as CD4<sup>+</sup> T-cells transduced with codon optimized and cysteine modified TCRs. These modifications should result in higher cell surface expression due to improved translation and improved preferential

pairing of the TCR-chains, but nevertheless CD8 $\alpha\beta$  co-expression was required for robust HLA-class I restricted helper functions.

Introduction of the CD8 $\alpha\beta$  co-receptor increased the sensitivity of the HLA-I-TCR td CD4<sup>+</sup> T cells approximately a 100 fold, leading to efficient recognition of target cells that express the antigen endogenously. Truncation of the intracellular domains of the CD8 $\alpha$  and CD8 $\beta$  subunits did not change the functional properties of the HLA-I-TCR td CD4<sup>+</sup> T-cells. Using CD4<sup>+</sup> T-cells transduced with several different HLA-I-TCRs we confirmed the generality of the data.

Although it was demonstrated that both for CD8 $\alpha$ [35] and CD8 $\beta$ [36] the intra- as well as the extracellular domain play a role in positive selection of thymocytes, we demonstrate that for the effector function of peripheral T-cells the CD8 $\alpha\beta$  co-receptor functions as an adhesive molecule rather than a signalling molecule. Therefore, to elicit robust helper functions in CD4<sup>+</sup> T-cells transduced with high-affinity HLA class I restricted TCRs introduction of the extracellular domains of CD8 $\alpha$  and  $\beta$  subunits

is required and sufficient. However, although we demonstrate equal proliferative capacity of HLA-I-TCR td CD4<sup>+</sup> T-cells co-expressing either  $\delta$ CD8 $\alpha\beta$  or wtCD8 $\alpha\beta$ , it needs to be studied in vivo whether they also demonstrate equal long-term persistence in vivo.

## Materials and Methods

### Construction of retroviral vectors and production of retroviral supernatant

TCR $\alpha$  and TCR $\beta$  chains of the HA-2-TCR[8], as well as of the codon optimized[37] and cysteine modified[38,39] next generation HA-2-TCR<sub>CC</sub>, HA-1-TCR<sub>CC</sub>[40], and PRAME-TCR<sub>CC</sub>[41] were linked using a self-cleaving T2A sequence[42] and combined with the truncated nerve growth factor (NGF-R) into a retroviral vector. All TCRs used were CD8 dependent and HLA-A\*0201-restricted. Both single vectors encoding unmodified CD8 $\alpha$  (wtCD8 $\alpha$ ), truncated CD8 $\alpha$  ( $\Delta$ CD8 $\alpha$ ), Lck mutated CD8 $\alpha$  (CD8 $\alpha$  Lck), unmodified CD8 $\beta$  (wtCD8 $\beta$ ) and truncated CD8 $\beta$  ( $\Delta$ CD8 $\beta$ ) as well as T2A linked wtCD8 $\alpha\beta$  and  $\Delta$ CD8 $\alpha\beta$  constructs were engineered.  $\Delta$ CD8 $\alpha$  consists of amino acids (aa) 1-190, Lck mutated CD8 $\alpha$  has mutations at position 201 and 203 (C $\rightarrow$ A), and  $\Delta$ CD8 $\beta$  consists of aa 1-176. CD8 $\alpha$  constructs were combined with the eGFP marker gene, whereas the CD8 $\beta$  constructs were combined with the NGF-R marker gene. T2A linked wtCD8 $\alpha\beta$  and  $\Delta$ CD8 $\alpha\beta$  constructs were engineered without a marker gene. Retroviral vectors encoding eGFP or NGF-R alone were used as control vectors (mock). Using the Moloney murine leukemia virus-based retroviral vector LZRS and packaging cells  $\phi$ -NX-A, viral supernatant was generated as previously described in detail[8].

### Flow cytometric analyses and cell sorting

For flow cytometric analyses as well as flow cytometry-based sorting, cells were labeled with tetramers for 1 h at 4°C or with mAbs directed against the various cell surface molecules for 30 minutes at 4°C. Cells were analyzed using the following mAbs: anti-CD3 APC-conjugated (Beckton Dickinson [BD], San Diego, CA, USA), anti-CD4 FITC-conjugated [BD], anti-NGF-R PE-conjugated [BD] or APC-conjugated (Cedarlane Laboratories, Hornby, Ontario, Canada), anti-BV2 PE-conjugated (Immunotech, Marseille, France) CD8 $\alpha$  FITC- [BD], APC- [BD] or PE-conjugated (Invitrogen, Paisley, UK) and CD8 $\beta$  PE-conjugated (Beckman Coulter, Fullerton, CA, USA). For cumulative measurement of several intracellular cytokines, the following mAbs were used: APC-labeled IFN- $\gamma$  or IL-2 mAb or PE-labeled TNF- $\alpha$  or CD40L mAb [BD].

PE-labeled and APC-labeled HA-2 peptide-HLA tetramers were produced as described previously[43]. For data acquisition, a FACSCalibur was used and for data analysis FlowJo software was used.

### Donor CD4<sup>+</sup> T-cells, stimulation and transduction

After study approval of the Leiden University Medical Center institutional review board and written informed consent according to the Declaration of Helsinki, peripheral blood mononuclear cells (PBMC) were obtained from hereditary hemochromatosis patients. From these PBMC samples, CMV-pp65-specific CD4<sup>+</sup> T-cells were isolated as previously described[44]. Briefly: PBMC were stimulated with 2  $\mu$ g/ml pp65-KYQEFFWDANDIYRI (pp65 peptide; HLA-DRB1\*0101-binding) peptide, and after 4 hours (h) of stimulation, IFN- $\gamma$  secreting CD4<sup>+</sup> T cells were isolated

using the IFN- $\gamma$  secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, CMV-specific CD4<sup>+</sup> T-cells were further purified by cell sorting based on TCR-BV2-staining. CMV-specific CD4<sup>+</sup> T-cell line was stimulated with irradiated (30 Gy) allogeneic PBMCs (1 $\times$ 10<sup>6</sup> cells/ml), and 800 ng/ml PHA (Murex Biotec Limited, Dartford, UK), and transduced 2 days after stimulation as described previously[45] with HA-2-TCR or empty vectors combined with NGF-R. After cell sorting based on NGF-R marker gene expression, NGF-R marker gene positive cells were re-stimulated, transduced with the different CD8 constructs, and sorted on basis of NGF-R expression, CD4 and CD8 cell surface expression. In addition, >94% MACS-enriched (Miltenyi Biotec) CD4<sup>+</sup> T-cells derived from total PBMCs were transduced with either next generation HA-1-, HA-2-, or PRAME-TCR or empty vectors combined with NGF-R in combination with the different CD8 co-receptor subunits, and these T-cells were used in experiments without further purification.

### Analysis of cytokine production and proliferation

To test the capacity of T-cells to specifically proliferate in response to antigen, a PKH (St. Louis, Missouri, USA) based assay was used[46]. T-cells were labeled with PKH-26 (St. Louis, Missouri, USA) according to manufacturer's instructions, and 1 $\times$ 10<sup>4</sup> T-cells were stimulated with 3 $\times$ 10<sup>4</sup> target cells. PKH dilution was analyzed using flow cytometry at day 5 after stimulation.

To analyze reactivity of TCR td CMV-specific CD4<sup>+</sup> T-cells, 5 $\times$ 10<sup>3</sup> td T-cells were cocultured with 2 $\times$ 10<sup>4</sup> target cells and after overnight incubation specific IFN- $\gamma$  production was measured by standard ELISA[40]. For cumulative measurement of several intracellular cytokines, 1 $\times$ 10<sup>5</sup> T-cells were stimulated with 2 $\times$ 10<sup>5</sup> EBV-transformed lymphoblastoid cell lines (LCLs) in the presence of 10  $\mu$ g/ml brefeldin A (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands), and 5 h after stimulation cytokine production was measured as previously described[44]. Targets used were HLA-typed LCL IZA (HLA-A\*0201<sup>+</sup> HA-2<sup>-</sup>), LCL JYW (HLA-A\*0201<sup>+</sup> HA-2<sup>+</sup>), LCL MRJ (HLA-A\*0201<sup>+</sup> HA-1<sup>+</sup> HA-2<sup>+</sup>), or LCL CBH (HLA-DRB1\*0101<sup>+</sup>) either unpulsed, or pulsed for 1 h at 37°C with 1  $\mu$ g/ml HLA-DRB1\*0101-binding pp65 peptide, or HLA-A2 binding HA-2 peptide (YIGVEVLVSV), or HA-1 peptide (VLHDDLLEA). In addition, HLA-A\*0201<sup>+</sup> PRAME<sup>-</sup> and HLA-A\*0201<sup>+</sup> PRAME<sup>+</sup> melanoma cells either unpulsed or pulsed with HLA-A\*0201 binding PRAME peptide (SLLQHLIGL) were used as targets. All tests were performed in duplo.

### Statistics

Experimental data was evaluated in a paired fashion by use of the students' T-test. Reported *P* values are 2-sided and were considered statistically different if <0.05.

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### Author Contributions

Conceived and designed the experiments: MVL RSH RDB JHFF MHMH. Performed the experiments: MVL RSH RDB. Analyzed the data: MVL RSH RDB JHFF MHMH. Wrote the paper: MVL JHFF MHMH.

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