Extracellular Domains of CD8α and CD8β Subunits Are Sufficient for HLA Class I Restricted Helper Functions of TCR-Engineered CD4⁺ T Cells

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Abstract

By gene transfer of HLA-class I restricted T-cell receptors (TCRs) (HLA-I-TCR) into CD8⁺ 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 CD8β molecule has to be co-transferred into the CD4⁺ T-cells to engineer optimal helper T-cells. In this study, we set out to determine the minimal part of CD8αβ needed for optimal co-receptor function in HLA-I-TCR transduced CD4⁺ T-cells. For this purpose, we transduced human peripheral blood derived CD4⁺ 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 CD8βα co-receptor in HLA-I-TCR transduced CD4⁺ T-cells behaves as an adhesion molecule, since for optimal antigen-specific HLA class I restricted CD4⁺ T-cell reactivity the extracellular domains of the CD8α and β subunits are sufficient.

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Introduction

Adaptive 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⁺ 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 CD8α⁺ immune responses in vivo, however, antigen-specific CD4⁺ T cells may play an essential role[14,15]. By TCR engineering of CD8α⁺ as well as CD4⁺ 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 CD8α molecule has to be co-transferred into the CD4⁺ T-cells to engineer optimal helper T-cells[16,17].

The CD8 molecule can be expressed as an αα or an αβ dimer, but is on peripheral TCRαβ T-cells mostly expressed as an αβ dimer[18–23]. The α subunit of CD8 binds to the non-polymorphic residues of the α3 domain of HLA class I, thereby enhancing the avidity of the TCR/MHC complex[24]. The cytoplasmic tail of the α subunit directly associates with the protein tyrosine kinase Lck[25–28], promoting signal transduction after T-cell activation. The intracellular domain of the β subunit enhances the association of CD8α 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 CD8αβ heterodimers bind MHC class I molecules more avidly than CD8αα homodimers[32–34].

Previously, it was reported that for optimal proliferation, cytokine production and cytotoxicity of HLA-I-TCR td CD4⁺ T-cells co-expression of CD8αβ was needed whereas co-expression of CD8αα only marginally increased functional activity[16,17]. Here, we studied whether the extracellular and/or intracellular part of CD8α and CD8β were required for this increased functional activity.

Results and Discussion

Extracellular CD8α and β are required and sufficient to elicit HLA class I restricted IFN-γ production

To verify that functional activity of high-affinity HLA-I-TCR transduced (td) CD4⁺ T-cells was improved by the transfer of CD8αα or CD8αβ co-receptor, CMV-specific CD4⁺ T-cells were transduced with the high-affinity HA-2-TCR with either only CD8α or with both the CD8α and CD8β subunits and purified based on CD8αα or CD8αβ 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⁺ LCLs stimulating the introduced HA-2-TCR, and antigen-specific IFN-γ production was measured (Figure 1A). As can be observed, HA-2-TCR td CMV-specific CD4⁺ T-cells co-transferred with CD8αα, CD8αβ or negative for CD8α 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 CD8αβ co-transferred T-cells were able to recognize...
extracellularly and presented HA-2 (Figure 1A). These results confirmed previous studies demonstrating that retroviral introduction of CD8β increased the functional activity mediated via the introduced TCR of the TCR td CD4+ T-cells[16,17].

To more precisely determine the part of the CD8β co-receptor responsible for increased functional activity of the HLA-A1-TCR td CD4+ T-cells, we constructed intracellularly truncated CD8α (ΔCD8α), Lck mutated CD8β (CD8α Lck), and intracellularly truncated CD8β (ΔCD8β). The HA-2-TCR td CMV-specific CD4+ T-cells were transduced with the different CD8α and CD8β constructs, purified based on CD8α or CD8β expression, and used as effector T-cells in the experiments described here above (Figure 1B). Results were similar to the experiments with unmodified (wt)CD8α and wtCD8β co-transferred HA-2-TCR td CMV-specific CD4+ T-cells (Figure 1A). Only TCR td CD4+ T-cells co-transferred with both CD8α and CD8β produced significant amounts of IFN-γ after stimulation with HA-2+ LCLs, irrespective of whether the co-transferred CD8α and β subunits were intracellularly truncated or whether the Lck binding motif of the CD8β subunit was mutated (Figure 1B). Mock td CMV-specific CD4+ T cells specifically produced IFN-γ only after stimulation with peptide pulsed LCLs (data not shown). These results demonstrate that for optimal HLA class I restricted IFN-γ production of the TCR td CD4+ T-cells, co-transfer of the extracellular domains of CD8α and β is required but that the intracellular domains can be disregarded.

Polyfunctionality of CD4+ T cells is important for optimal helper function. Therefore, we studied the capacity of HA-2-TCR td CMV-specific CD4+ T cells co-transferred with different CD8 constructs to produce not only IFN-γ, but also TNF-α and IL-2 and upregulate CD40L (Figure 1C). TCR td CD4+ T cells co-transferred with either wtCD8α or ΔCD8β produced significantly more cytokines and demonstrated significantly more CD40L upregulation after stimulation with either HA-2 pulsed or HA-2+ LCLs (p<0.05 indicated with asterisks) than CD8α negative or CD8βα expressing TCR td CD4+ T-cells. No significant difference in cytokine production or CD40L upregulation after HA-2 specific stimulation was observed between TCR td CD4+ T-cells co-transferred either with wtCD8αβ or ΔCD8β. The results in Figure 1C demonstrate that for IFN-γ, TNF-α and IL-2 production as well as for CD40L upregulation after stimulation with HA-2+ LCLs co-transfer of CD8α and β, and most importantly the extracellular domains of these CD8 subunits, is required.

In conclusion, to generate polyfunctional HA-2-TCR td CMV-specific CD4+ T-cells, co-transfer of both CD8α and β is required, but the intracellular domains of these CD8 subunits can be dismissed.

ΔCD8α and ΔCD8β improve HLA-class I restricted avidity similarly efficiently as wtCD8α and wtCD8β

To analyze whether TCR td CD4+ 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+ T-cells (Figure 1D and E). No specific HA-2 tetramer staining could be observed for mock and TCR td CD4+ T-cells without CD8β co-transfer (data not shown). However, co-transfer of CD8α alone or transfer of CD8βα 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+ T-cells were co-transferred with multistronic vectors in which the CD8α and CD8β molecules were linked with a 2A sequence resulting in equimolar levels of both CD8α and β 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+ T-cells co-transferred with the multistronic CD8αβ vectors, whereas TCR td CD4+ T-cells co-transferred with the wtCD8αβ or ΔCD8βΔα multistronic 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 wtCD8αβ or ΔCD8βΔα equally improved the function of CD4+ T cells transduced with a next generation HA-2-TCR,Cα, that was codon optimized and cysteine modified to improve TCR cell surface expression. For this purpose, CMV-specific CD4+ T-cells were transduced with the next generation HA-2-TCR,Cα, either without or in combination with different CD8 subunits, purified using flow cytometry based cell sorting and tested for HA-2-specific IFN-γ 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+ target cells). As demonstrated in Figure 2A, the HA-2-TCR,Cα td CD4+ T-cells expressing either wtCD8αβ or ΔCD8βΔα were equally reactive against HA-2 peptide loaded target cells, and approximately a 100 fold more sensitive compared to CD8 negative or CD8βα expressing HA-2-TCR,Cα td CD4+ T-cells. In addition, whereas CD8 negative or CD8βα expressing HA-2-TCR,Cα td CD4+ T-cells demonstrated no or low reactivity against HA-2+ target cells, the ΔCD8βα and wtCD8βα expressing HA-2-TCR,Cα td CD4+ T-cells were highly reactive against HA2+ target cells. To confirm the significantly increased sensitivity of the wtCD8αβ and ΔCD8βΔα expressing HA-2-TCR,Cα td CD4+ T-cells compared to the CD8 negative or CD8βα expressing cells, we performed a proliferation assay in which we stimulated PKH-labeled T-cells with HA-2+ and HA-2- target cells and analyzed proliferation by measuring PKH dilution at day 5 after stimulation. As can be observed in Figure 2B, wtCD8αβ and ΔCD8βΔα HA-2-TCR,Cα td CD4+ T-cells proliferated equally efficiently after stimulation with HA-2+ target cells, whereas for the CD8 negative or CD8βα 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+ T-cells for HLA-A2+ HA-2+ target cells approximately a 100-fold. This increase in avidity is necessary to elicit efficient IFN-γ production and proliferation of HA-2-TCR and HA-2-TCR,Cα td CD4+ T-cells after stimulation with physiologically relevant levels of HA-2.

In general, HLA-1-TCR td CD4+ T-cells require co-transfer of only the extracellular CD8αβ domains

To confirm the generality of these data, polyclonal peripheral CD4+ 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+ T cells transduced with either HA-1-TCR,Cα, HA-2-TCR,Cα or PRAME-TCR,Cα displayed IFN-γ-, IL-2- and TNF-α production only after stimulation with peptide pulsed target cells (1 μg/mL), and not after stimulation with antigen-pulsed target cells expressing endogenously processed antigen. Introduction of only the wtCD8α molecule induced some cytokine production against peptide pulsed target cells and antigen-positive target cells. However, when wtCD8αβ or ΔCD8βΔα were introduced, substantial percentages of transduced T-cells produced IFN-γ-, IL-2 and TNF-α 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 CD8α and β for HLA-class I restricted helper functions.
Extracellular CD8αβ Co-Receptor Domains Essential
Figure 1. HLA-I-TCR td CD4+ T-cells co-transferred with wtCD8αβ or intracellularly modified CD8αβ demonstrate equal effector functions. To study the minimal part of CD8 needed for optimal co-receptor function in HLA-I-TCR td CD4+ T-cells, HA-2-TCR td CMV-specific CD4+ T-cells (A) co-transferred with wtCD8α or wtCD8αβ co-receptor, or (B) co-transferred with either wtCD8α, ΔCD8β or CD8α Lck in combination with either wtCD8β or ΔCD8β were purified and used in a stimulation assay. Td T-cell populations were tested against HLA-DR1+ LCL-CBH either un pulsed (grey striped bars) or pulsed with pp65 peptide (grey bars), or against HLA-A2+ HA-2+ LCL-JY (black bars), IFN-γ production was measured after 18 h of stimulation in duplicate, and a representative experiment out of 3 is depicted. The IFN-γ production of the different CD8αβ expressing TCR td T-cells was compared to the IFN-γ production of CD8αβ 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+ T-cells, both mock and HA-2-TCR td CMV-specific CD4+ T-cells with or without co-transfer of different CD8 subunits as indicated in the figure were stimulated with HLA-DR1+ LCL-CBH pulsed with pp65 peptide (grey bars; pp65 pep), unpulsed HLA-A2+ HA-2+ LCL-IZA (white bars; control), HA-2 peptide pulsed HLA-A2+ HA-2+ LCL-IZA (black bars; HA-2 pep) or HLA-A2+ HA-2+ LCL-JY (striped bars; HA-2 endogenous). After 5 h of stimulation, T-cells were stained with anti-IFN-γ, anti-TNF-α, anti-CD40L and anti-IL-2 mAbs and were analyzed using flow cytometry. The percentage of IFN-γ, TNF-α and IL-2 producing or CD40L expressing T-cells after stimulation is depicted. The percentages of cytokine producing and CD40L upregulating CD8αβ expressing TCR td T-cells that were significantly higher than CD8 negative and CD8αβ 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+ T-cells co-transferred with the different CD8α and CD8β constructs, HA-2 tetramer staining was analyzed. (D) Mock or (E) HA-2-TCR td CD4+ T-cells co-transferred with either wtCD8α+T2A-wtCD8β (wtCD8 T2A; left dot plots) or ΔCD8α+T2A-wtCD8β (ΔCD8β T2A, right dot plots) were stained with anti-CD8α and β mAbs and HA-2-tetramers and analyzed using flow cytometry. A representative example of 2 independent experiments is depicted. doi:10.1371/journal.pone.0065212.g001

Figure 2. Improved HLA-class I restricted avidity of CD8αβ expressing HA-2-TCR td CD4+ T-cells results in improved proliferation. (A) To study whether co-transfer of CD8 would also improve the peptide sensitivity of CD4+ T-cells transduced with a next generation HA-2-TCR, both mock and HA-2-TCR td CMV-specific CD4+ 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+ HA-2+ LCL IZA (white bars; LCL IZA), HLA-A2+ HA-2+ LCL-IZA pulsed with decreasing concentrations of HA-2 peptide (range 1 μM-10 nM) or HLA-A2+ HA-2+ LCL JWY (striped bars; LCL JWY). IFN-γ production was measured after 18 h of stimulation in duplicate, and a representative experiment out of 2 is depicted. The IFN-γ production of ΔCD8β and wtCD8αβ expressing HA-2-TCR td CD4+ T-cells significantly higher (p-values <0.05) than CD8 negative or CD8αβ expressing HA-2-TCR.CD4+ T-cells is indicated with an asterisk. (B) To investigate their proliferative capacity, both mock and HA-2-TCR td CD4+ T-cells without CD8 or co-transferred with wtCD8α, wtCD8β, or ΔCD8β were purified based on markergene expression and CD8 cell surface expression and were either not stimulated (filled histograms) or stimulated with HLA-A2+ HA-2+ LCL-JY (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
Conclusions

We hypothesize that CD8 co-transfer in antigen-experienced CD4+ T-cells potentially poses the risk of overstimulation. To minimize the risk of overstimulation in HLA class I restricted TCR transduced CD4+ 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+ T-cells co-expression of CD8αβ co-receptors is superior to CD8αα co-receptors, and CD8αβ co-expressing T-cells were superior in producing IFN-γ, TNF-α, and IL-2, in upregulating CD40L, and in antigen specific proliferation. Expression of the extracellular domains of CD8αβ was required both for CD4+ T-cells transduced with unmodified HA-2-TCR, as well as CD4+ 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 CD8αβ co-expression was required for robust HLA-class I restricted helper functions.

Introduction of the CD8αβ co-receptor increased the sensitivity of the HLA-I-TCR td CD4+ 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 CD8α and CD8β subunits did not change the functional properties of the HLA-I-TCR td CD4+ T-cells. Using CD4+ T-cells transduced with several different HLA-I-TCRs we confirmed the generality of the data.

Although it was demonstrated that both for CD8αβ[35] and CD8αβ[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 CD8αβ co-receptor functions as an adhesive molecule rather than a signalling molecule. Therefore, to elicit robust helper functions in CD4+ T-cells transduced with high-affinity HLA class I restricted TCRs introduction of the extracellular domains of CD8α and β subunits.

Figure 3. In general, co-transfer of the extracellular domains of CD8α and β is required and sufficient. To confirm the generality of the previous data, total CD4+ 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+ T-cells were stimulated and tested for cytokine production using flow cytometry. HA-1- or HA-2-TCR td CD4+ T-cells were stimulated either with HA-1 or HA-2 peptide pulsed or unpulsed HLA-A2+ HA-1+ HA-2+ LCL-IZA, or HLA-A2+ HA-1+ HA-2+ LCL-MRJ, and PRAME-TCR td CD4+ T-cells were stimulated either with PRAME peptide pulsed or unpulsed HLA-A2+ PRAME+ melanoma cells, or HLA-A2+ PRAME+ melanoma cells. 5 h After stimulation, T-cells were permeabilized and stained with anti-NGF-R in combination with either anti-IFN-γ (upper panel), anti-IL-2 (middle panel) or anti-TNF-α (lower panel), and analyzed using flow cytometry. The percentage of marker gene 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.

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Conclusions

We hypothesize that CD8 co-transfer in antigen-experienced CD4+ T-cells potentially poses the risk of overstimulation. To minimize the risk of overstimulation in HLA class I restricted TCR transduced CD4+ 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+ T-cells co-expression of CD8αβ co-receptors is superior to CD8αα co-receptors, and CD8αβ co-expressing T-cells were superior in producing IFN-γ, TNF-α, and IL-2, in upregulating CD40L, and in antigen specific proliferation. Expression of the extracellular domains of CD8αβ was required both for CD4+ T-cells transduced with unmodified HA-2-TCR, as well as CD4+ 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 CD8αβ co-expression was required for robust HLA-class I restricted helper functions.

Introduction of the CD8αβ co-receptor increased the sensitivity of the HLA-I-TCR td CD4+ 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 CD8α and CD8β subunits did not change the functional properties of the HLA-I-TCR td CD4+ T-cells. Using CD4+ T-cells transduced with several different HLA-I-TCRs we confirmed the generality of the data.

Although it was demonstrated that both for CD8αβ[35] and CD8αβ[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 CD8αβ co-receptor functions as an adhesive molecule rather than a signalling molecule. Therefore, to elicit robust helper functions in CD4+ T-cells transduced with high-affinity HLA class I restricted TCRs introduction of the extracellular domains of CD8α and β subunits.
is required and sufficient. However, although we demonstrate equal proliferative capacity of HLA-I-TCR td CD4+ T-cells co-expressing either ΔCD8ß or wtCD8ß, 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**

TCRα and TCRβ chains of the HA-2-TCR[8], as well as of the 39-mer optimized[37] and cystine modified[38,39] next generation HA-2-TCRCC, HA-1-TCRCC[40], and PRAME-TCRCC[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 CD8β dependent and HLA-A*0201 restricted. Both single vectors encoding unmodified CD8β (wtCD8β), truncated CD8β (∆CD8β), Lck mutated CD8β (CD8β Lck), unmodified CD8β (wtCD8β) and truncated CD8β (∆CD8β) as well as T2A linked wtCD8β and ∆CD8β constructs were engineered. ∆CD8β consists of amino acids (aa) 1-190, Lck mutated CD8β consists of aa 1-176. wtCD8β and ∆CD8β constructs were engineered without a NGF-R 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 pseudotyped LCL IZA and packaging cells φ-NN-X, 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) CD8β FITC- [BD], APC- [BD] or PE-conjugated (Invitrogen, Paisley, UK) and CD8β PE-conjugated (Beckman Coulter, Fullerton, CA, USA). For cumulative measurement of several intracellular cytokines, 1×10^5 T-cells were transduced with 2×10^5 EBV-transformed lymphoblastoid cell lines (LCLs) in the presence of 10 μg/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+ HA-2+), LCL JYW (HLA-A*0201+ HA-2+), LCL MRJ (HLA-A*0201+ HA-1+ HA-2+), or LCL CBI (HLA-DRB1*0101) either unpulsed, or pulsed for 1 h at 37°C with 1 μg/mL HLA-DRB1*0101-binding pp65 peptide, or HLA-A2 binding HA-2 peptide (YIGVEVLVS), or HA-1 peptide (VLHDDDLLE). In addition, HLA-A*0201+ PRAME and HLA-A*0201+ PRAME melanoma cells either unpulsed or pulsed with HLA-A*0201 binding PRAME peptide (SLIQHLIGL) 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 P<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.
