RESEARCH PAPER

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Human MHC Class I-restricted high avidity CD4+ T cells generated by co-transfer of TCR and CD8 mediate efficient tumor rejection in vivo

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In this study, we generated human MHC Class I-restricted CD4+ T cells specific for Epstein-Barr virus (EBV) and cytomegalovirus (CMV), two herpesviridae associated with lymphoma, nasopharyngeal carcinoma and medulloblastoma, respectively. Retroviral transfer of virus-specific, HLA-A2-restricted TCR-coding genes generated CD4+ T cells that recognized HLA-A2/peptide multimers and produced cytokines when stimulated with MHC Class II-deficient cells presenting the relevant viral peptides in the context of HLA-A2. Peptide titration revealed that CD4+ T cells had a 10-fold lower avidity than CD8+ T cells expressing the same TCR. The impaired avidity of CD4+ T cells was corrected by simultaneously transferring TCR- and CD8-coding genes. The CD8 co-receptor did not alter the cytokine signature of CD4+ T cells, which remained distinct from that of CD8+ T cells. Using the xenogeneic NOD/SCID mouse model, we demonstrated that human CD4+ T cells expressing a specific TCR and CD8 can confer efficient protection against the growth of tumors expressing the EBV or CMV antigens recognized by the TCR. In summary, we describe a robust approach for generating therapeutic CD4+ T cells capable of providing MHC Class I-restricted immunity against MHC Class II-negative tumors in vivo.

Introduction

The transfer of genes coding for T-cell receptor (TCR) α and β chains into primary T cells is an effective strategy to rapidly generate high numbers of antigen-specific T cells for adoptive cell therapy.[1–11] Major advantages of this approach include that it can be used in cancer patients who are unable to mount effective T-cell responses, and that it can involve TCR specificities that are absent in the patient repertoire.[12,13] The clinical efficacy of monoclonal TCR gene therapy has recently been demonstrated in melanoma and synovial cell sarcoma patients.[14] To date, all TCR α and β chain-coding genes used in clinical trials and the majority of TCR used in preclinical models have been derived from MHC Class I-restricted CD8+ T cells. This is partly due to the historical focus on the identification of cancer epitopes recognized by CD8+ cytotoxic T cells, which have the potential to protect against a large number of tumors expressing MHC Class I. Conversely, only a few neoplasms express MHC Class II molecules, which are required for recognition by CD4+ T cells. However, a recent clinical trial demonstrated the therapeutic efficacy of adoptively transferred CD4+ T cells in a melanoma patient,[15] and similar studies in a murine model indicate that melanoma-specific CD4+ cells may provide more effective tumor immunity than CD8+ T cells.[16] Such preclinical studies demonstrated that the protection by CD4+ T cells is dependent upon induction of MHC Class II expression by melanoma cells in vivo, indicating that tumors that do not upregulate MHC Class II may escape CD4+ T cell-mediated immunity.

Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are two herpesviridae that establish a chronic infection in a large proportion of individuals.[17] There is clear evidence that EBV is involved in several malignancies including Hodgkin's lymphoma, nasopharyngeal carcinoma and lymphoproliferative diseases in immunosuppressed individuals. All these tumors typically express the EBV-encoded protein LMP2, which has been exploited as a target for adoptive T-cell therapy.[18–20] Although the link between chronic CMV infection and cancer is more controversial, there is now strong evidence that latent CMV is present in a large proportion of medulloblastoma tumors.[21] High CMV

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load in tumor samples has been associated with poor prognosis, and treatment with the antiviral drug ganciclovir has been shown to limit the growth of CMV-positive medulloblastoma tumors in xenogenic settings. The CMV protein pp65, which is expressed by these tumors, provides an attractive target for the development of immunotherapeutic approaches against medulloblastoma.

The aim of this study was to optimize CD4+ T-cell immunity against EBV and CMV epitopes that are normally recognized by CD8+ T cells. Using two MHC Class I-restricted TCRs specific for LMP2 and pp65, we demonstrated that gene transfer into CD4+ T cells generated helper T cells with lower functional avidity than CD8+ T cells expressing the same virus-specific TCRs. However, the co-transfer of TCR- and CD8-coding genes generated high avidity CD4+ T cells that retained the antigen-specific cytokine profile of "helper" T cells. Adoptive therapy experiments indicated that the efficacy of high avidity CD4+ T cells in providing protective tumor immunity was similar to the therapeutic efficacy seen with CD8+ T cells. Together, our findings indicate that the transfer of TCR- plus CD8-coding genes is a robust strategy to optimize CD4+ T-cell responses against human MHC Class II-negative cancers.

Results

MHC Class I-restricted TCR specific for EBV and CMV are functional in CD4+ T cells. In this study, we used two TCRs specific for well-defined CD8+ T-cell epitopes in the LMP2 antigen of EBV and in the pp65 protein of CMV. The EBV-targeting TCR-coding genes were isolated from an HLA-A2-restricted CD8+ T-cell clone specific for the LMP2 peptide CLGGLLTMV (pCLG),[22] and the CMV-targeting TCR-coding genes were synthesized using public sequence data relative to the TCR specific for the HLA-A2-presented pp65 peptide NLVPMVATV (pNLV).[23] The sequences coding for TCR α and β variable fragments were codon optimized and linked to sequences encoding murine constant regions that were engineered to contain an additional disulphide bond (**Fig. S1A**). These modifications were chosen because previous studies demonstrated that they enhance TCR expression in human T cells while reducing mispairing between introduced and endogenous TCR chains.[24–28]

Primary human T cells were activated with anti-CD3 antibodies and then transduced with retroviral vectors encoding EBV- or CMV-targeting TCRs. Antibodies specific for the murine constant β chain were used to detect TCR expression, and HLA-A2/peptide multimers were used to determine whether expressed TCR bound to pCLG and pNLV. As shown in **Figure 1A and B**, both the EBV- and the CMV-targeting TCRs were expressed by CD8+ T cells as well as CD4+ T cells. Moreover, the majority of T cells that stained with the anti-murine constant β chain also bound the EBV pentamer and the CMV tetramer, demonstrating that introduced TCRs displayed the expected virus specificity. The multimer staining was not limited to CD8+ T cells, but was also detectable in CD4+ T cells (identified as the CD8-negative T cells in the bottom panels), indicating that the specific binding of the HLA-A2/peptide multimers can occur in the absence of the CD8 co-receptor.

In order to assess the functional activity of EBV- or CMV-targeting TCRs, freshly transduced human T cells were stimulated with pCLG and pNLV, respectively, followed by intracellular staining for interleukin (IL)-2, intereferon γ (IFNγ) and tumor necrosis factor α (TNFα). CD8+ T cells exhibited a strong cytokine production upon stimulation with TCR-recognized, but not control, peptides (**Fig. 1C and E**). Similarly, CD4+ T cells responded to the cognate peptides with robust cytokine production (**Fig. 1D and F**). The analysis of freshly transduced T cells consistently revealed a more efficient peptide-specific IL-2 production by CD4+ T cells than by CD8+ T cells. Taken together, these data indicate that both EBV- and CMV-targeting TCRs are functionally active in CD8+ and CD4+ T cells. It is worth noting that T2 cells used for peptide presentation expressed HLA-A2 but did not express HLA Class II molecules (**Fig. S2**), due to a deletion of the HLA Class II region on chromosome 6.[29] This indicates that the triggering of peptide-specific cytokine production by CD4+ T cells was HLA Class I-restricted and did not require the binding of CD4 to HLA Class II molecules.

HLA Class I-restricted CD4+ T cells have a lower functional avidity than CD8+ T cells. Next, we assessed the ability of HLA-Class I-restricted CD4+ T cells to recognize endogenously processed antigens. For these experiments, we used human leukemia K562 cells transfected to express HLA-A2 (referred to as KA2 cells) and either LMP2 or pp65. We selected the K562 system as not only it allowed us to assess HLA restriction and antigen-specificity in vitro, but also enabled us to perform antigen-specific tumor protection experiments in vivo, owing to the ability of K562 cells to form tumors in immunodeficient mice (see below). Furthermore, since K562 tumor cells did not express HLA-DR, DQ and DP (**Fig. S2**), it was possible to assess the function of engineered CD4+ T cells against tumor cells that lack HLA Class II molecules. Stimulation of transduced T cells revealed that the EBV- and CMV-targeting TCRs trigger cytokine production when stimulated with KA2 cells expressing LMP2 and pp65, respectively (**Fig. 2A and B**). Consistently, we found that the percentage of CD4+ T cells producing cytokines was lower compared than that of CD8+ T cells, prompting to explore the possible reasons for such a reduced activity of CD4+ T cells against tumor cells endogenously expressing target antigens.

We used peptide titration experiments to assess the functional avidity of CD4+ and CD8+ T cells expressing the same TCR. Transduced T cells expressing EBV- and CMV-targeting TCRs were stimulated with decreasing amount of pCLG and pNLV, respectively, followed by intracellular cytokine staining of gated CD4+ and CD8+ T cells. At 1 nM pCLG concentration, EBV-TCR-expressing CD8+ T cells mounted a robust IFNγ and IL-2 response, while a similar response by CD4+ T cells required 10 nM peptide concentration (**Fig. 2C**). The analysis of CMV-TCR-engineered T cells also revealed that the functional avidity of CD4+ T cells was at least 10-fold lower than that of CD8+ T cells (**Fig. 2D**). The reduced avidity of CD4+ T cells may explain their reduced ability to recognize tumor cells endogenously expressing TCR target antigens (**Fig. 2A and B**).

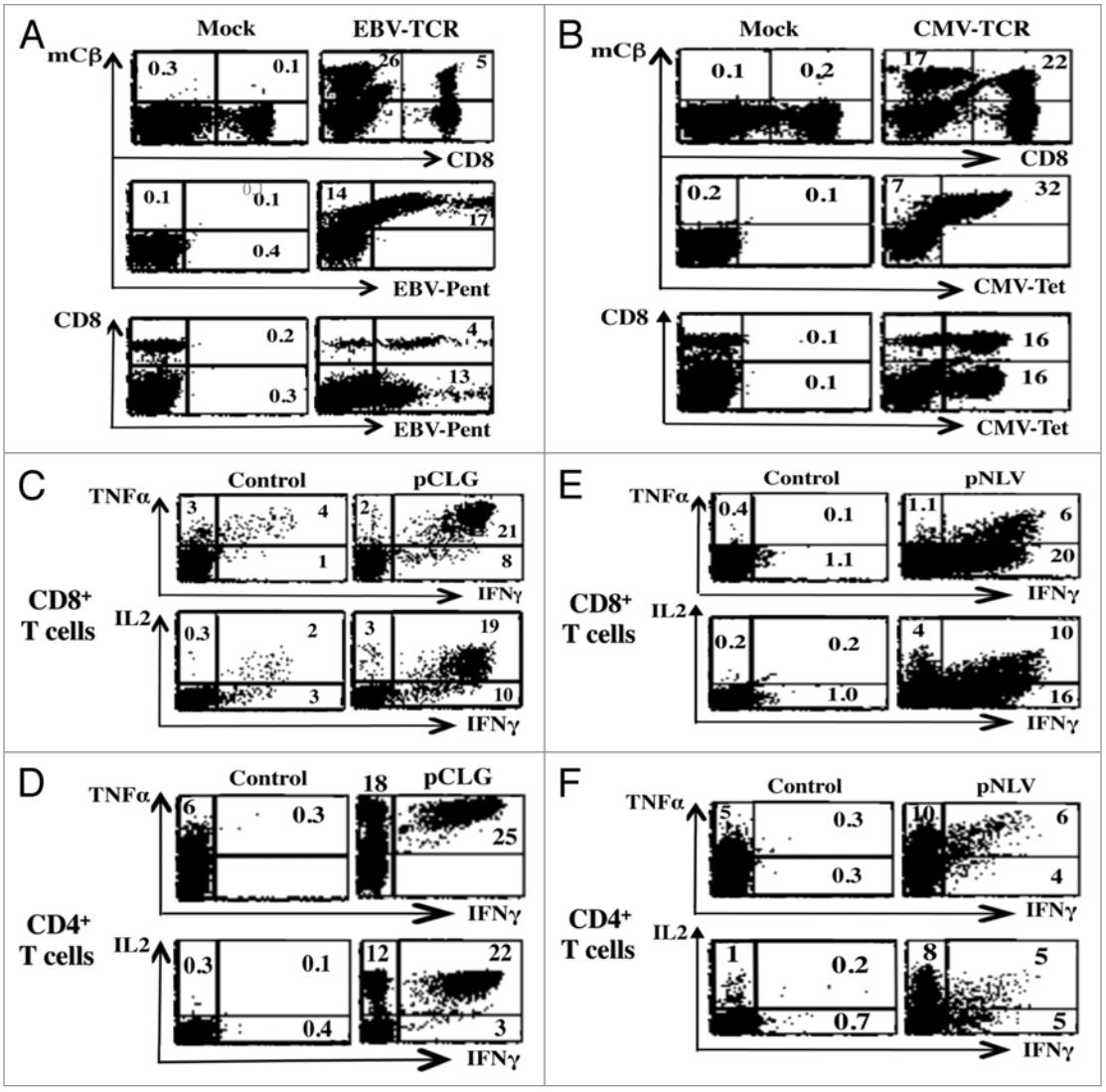

Figure 1. Generation of MHC Class I restricted-EBV- and CMV-specific CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ helper T (T_H) cells through TCR gene transfer. (**A and B**) Retroviral transduction of EBV-TCR (**A**) and CMV-TCR (**B**) into human peripheral blood mononuclear cells (PBMCs). Freshly transduced T cells were stained with anti-mCβ to monitor the expression of introduced TCR β chain, and with an EBV-pentamer or CMV-tetramer to monitor the expression of correctly paired TCR α/β chains. The numbers in quadrants refer to the percentage of relevant T cells in gated live cells after TCR transduction. (**C–F**) Cytokine production of EBV-TCR- (**C and D**) and CMV-TCR- (**E and F**) transduced cells, gated on CD8+ and on CD4+ populations as indicated. Freshly transduced T cells were stimulated overnight with control or specific peptides, followed by the analysis of intracellular cytokine production in gated CD8+ or CD4+ T-cell populations. The numbers in quadrants refer to the percentage of cytokine-producing T cells. Similar results were obtained in more than five independent experiments.

Transfer of the CD8 co-receptor into CD4+ T cells corrects their reduced avidity. Although the EBV- and CMV-targeting TCRs used here were "CD8-independent" in their ability to bind HLA-A2 multimers and produce cytokines upon peptide stimulation (**Fig. 1**), we hypothesized that the CD8 co-receptor is required for optimal recognition of low antigen concentration. In order to test this contention, we generated vectors containing both TCR-coding genes and genes encoding the CD8 α and β chains (**Fig. 3A**). Transduction experiments using primary human T cells demonstrated an efficient TCR- and CD8-coding gene transfer into CD4+ T cells. The analysis of bulk T cells transduced with the TCR/CD8-coding vector revealed that all T cells that expressed the exogenous TCR (as defined by multimer binding) also expressed CD8 (**Fig. 3B**). As expected, the CD4+ population of bulk T cells transduced with a control vector coding for the TCR (but not for CD8) exhibited TCR expression only (**Fig. 3B**).

Magnetic cell sorting was performed to assess antigen-specific cytokine secretion and cytotoxicity of transduced CD8+ T cells as well as of CD4+ cells transduced with the TCR only or with the TCR plus CD8. Typically, the purity of the CD4+ T cells was greater than 99%, and transduction with the TCR/CD8 vector resulted in CD8 expression in most of the purified CD4+ T cells (**Fig. 3C and D**). The purification of the CD8+ T cell population typically resulted in a purity of > 95%. Multimer staining was used to demonstrate that the majority of the purified T-cell

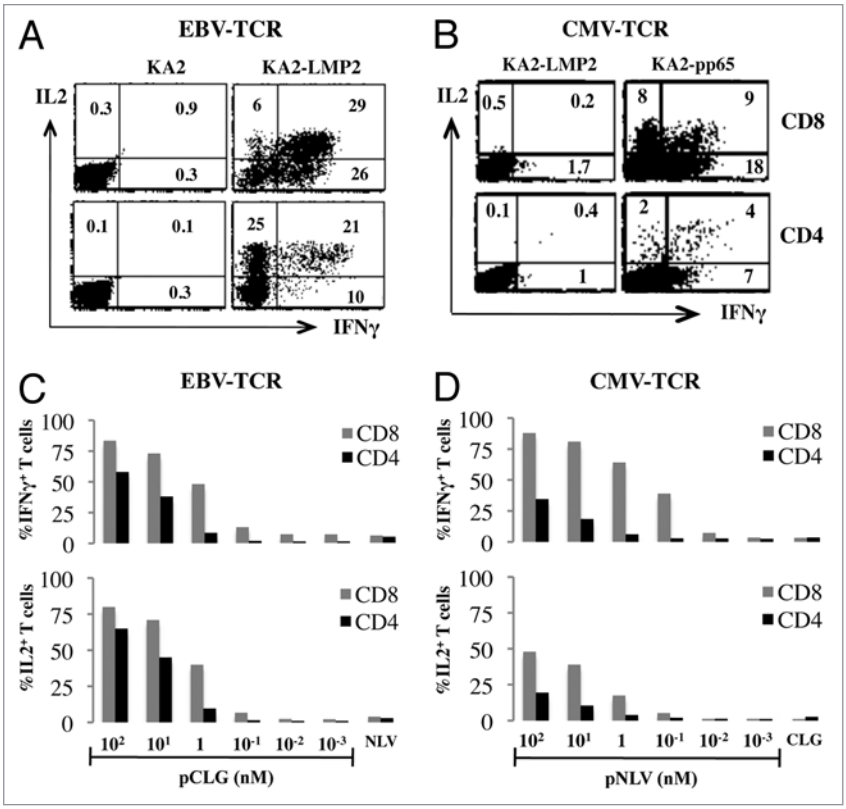

Figure 2. EBV-TCR- and CMV-TCR-engineered CD4+ T cells display a lower avidity than their CD8+ counterparts. (**A and B**) Recognition of endogenously presented antigens by EBV-TCR- and CMV-TCR-engineered T cells. EBV-TCR- and CMV-TCR transduced T cells were expanded using at least 3 rounds of stimulation with the appropriate peptide to obtain more than 80% TCR-positive T cells in both the CD8+ and CD4+ populations. EBV-specific (**A**) and CMV-specific (**B**) T cell lines were then stimulated overnight with the HLA-A2-transfected tumor cell line K562 (KA2) that had previously been transduced with constructs to express LMP2 (KA2-LMP2) or pp65 (KA2-pp65). After overnight stimulation, intracellular cytokine production was measured in gated CD8+ or CD4+ T cells. The numbers in quadrants refer to the percentage of cytokine-producing T cells. Data are representative of three independent experiments. (**C and D**) Comparison of intracellular cytokine production by CD8+ and CD4+ T cells. EBV-specific (**C**) and CMV-specific (**D**) T-cell lines were simulated overnight with decreasing concentrations of the relevant TCR-recognized peptides, or with a control peptide. The percentage of gated CD8+ or CD4+ T cells producing interferon γ (IFNγ) (top panels) and interleukin-2 (IL-2) (bottom panels) was determined by intracellular staining and is shown in gray for CD8+ T cells and in black for CD4+ T cells. Control stimulations were performed by using the highest concentration (100 nM) of control peptide. Data are representative of three independent experiments.

populations expressed the introduced EBV- (**Fig. 3C**) or CMV-targeting TCR (**Fig. 3D**).

Peptide titration experiments revealed that CD4+ T cells expressing both the EBV-targeting TCR and CD8 produced IFNγ in response to 1 nM peptide, a concentration that also triggered IFNγ production by CD8+ T cells (**Fig. 3E**). As previously observed, IFNγ production by CD4+ T cells expressing the EBV-targeting TCR only required a peptide concentration 10-fold higher. A similar 10-fold difference was seen when IL-2 production was analyzed in CD4+ T cells transduced with TCR only or with TCR plus CD8 (**Fig. 3E**). The analysis of CMV-TCR-transduced T-cell populations also showed that the TCR/CD8 co-transfer increases the avidity of CD4+ T cells to a level that is comparable to that of CD8+ T cells. This was most noticeable for IFNγ production, which was optimal for all T-cell populations analyzed at 1 μM peptide concentration. Conversely, the CD8 co-receptor was required for IFNγ production at peptide concentrations ≤ 1 nM (**Fig. 3F**).

Cytotoxicity experiments revealed that the co-transfer of TCR- and CD8-coding genes also improves the ability of CD4+ T cells to kill peptide-loaded target cells and tumor cells endogenously expressing TCR-recognized antigens (**Fig. 4A and B**). Both EBV-TCR- and CMV-TCR-engineered T cells responded to cognate peptides by upregulating granzyme B, a cytotoxic effector molecule. The peptide-specific granzyme B upregulation (from approximately 20% to 83% positive cells) was similar for CD8+ T cells and for CD4+ T cells transduced with the EBV-TCR only or coupled to CD8 (**Fig. 4C**). Conversely, granzyme B upregulation was more effective in CD8+ T cells transduced with the CMV-TCR as compared with CD4+ T cells expressing the CMV-TCR with or without CD8 (**Fig. 4D**).

Transfer of the CD8 co-receptor does not alter the cytokine profile of CD4+ T cells. The experiments above indicated that the CD8 co-receptor increases the avidity of TCR-transduced CD4+ T cells to a level that is comparable to that of CD8+ T cells. In order to explore whether the CD8 co-receptor would alter the biological function of CD4+ "helper" T cells to be more similar to that of CD8+ T cells, we used the luminex platform and simultaneously analyzed the antigen-specific production of 11 different cytokines. For this experiment, purified CD8+ T cells transduced with the TCR only or with the TCR plus CD8 were compared with purified CD4+ T cells subjected to the same genetic engineering. Such four T-cell populations were stimulated with KA2 cells endogenously expressing the TCR-recognized LMP2 antigen, or with KA2 control cells to measure non-specific cytokine production. **Figure 5** illustrates the production of 11 cytokines by the two CD8+ T-cell populations (transduced with TCR or TCR plus CD8) and by the two CD4+ populations (transduced with TCR or TCR plus CD8). While the antigen-specific production of IFN-γ, IL-2 and IL-8 was similar in all four T-cell populations, there was a trend for increased IL-6 and IL-1β production by CD4+ T cells. More striking was the difference in TNFα, IL-4 and IL-5 production: CD4+ T cells showed indeed a robust production of these cytokines, while CD8+ T cells produced only background levels. Most importantly, the CD8 co-receptor did not change the antigen-specific cytokine profile of engineered CD4+ T cells.

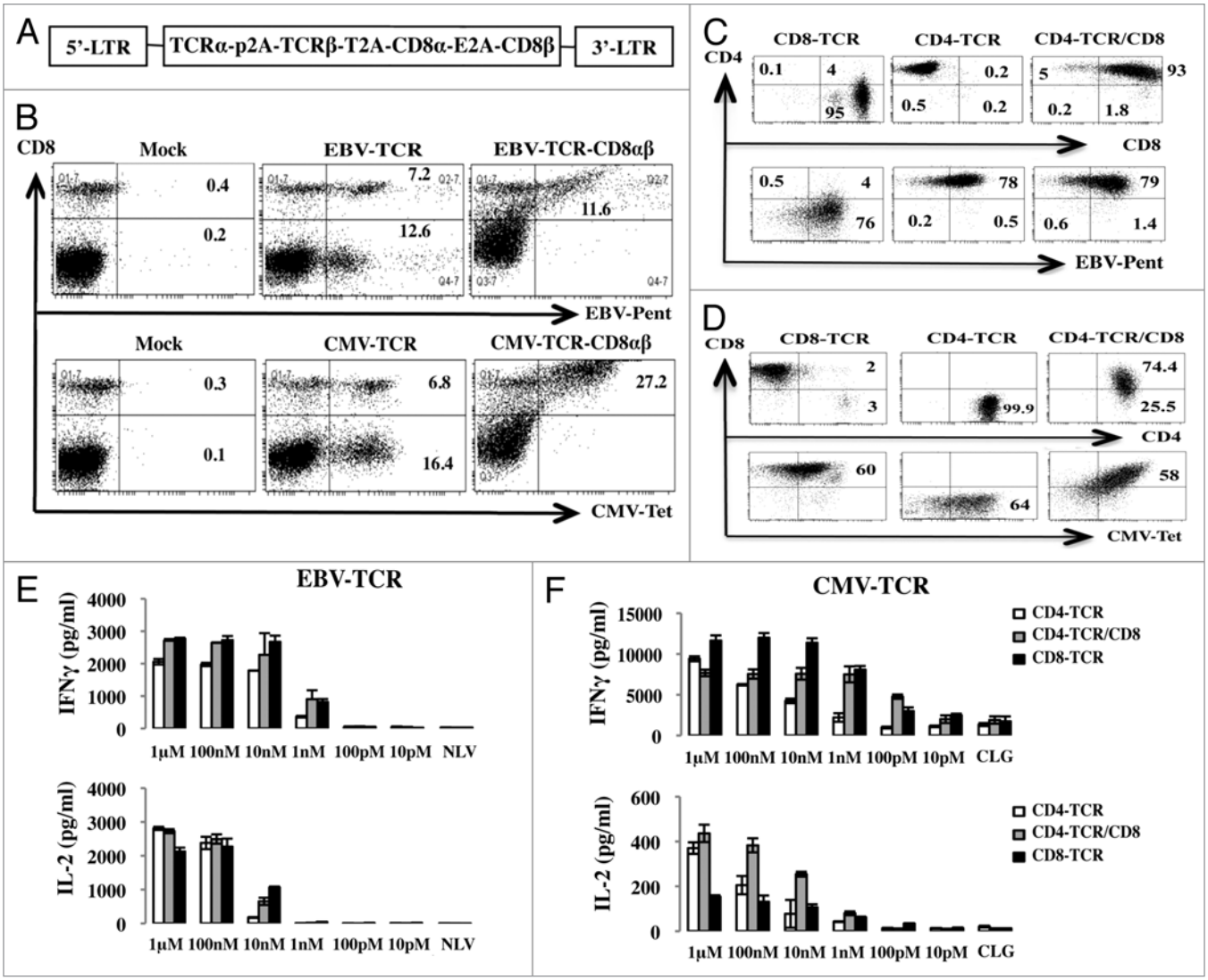

Figure 3. Introduction of CD8α/β can improve the avidity of TCR-engineered CD4+ T cells. (**A**) Schematic diagram showing the molecular structure of the retroviral vector containing TCR αβ- and human CD8 αβ-conding genes linked by distinct 2A sequences. (**B**) Expression of the TCR/CD8 constructs when transduced into human peripheral blood mononuclear cells (PBMCs). PBMCs were mock treated or transduced with TCR only or with TCR plus CD8 as indicated on the top of each panel. Freshly transduced T cells were stained with anti-CD8 antibodies and the corresponding multimers. The numbers in quadrants refer to the percentage of EBV-pentamer or CMV-tetramer positive cells. (**C and D**) Freshly EBV-TCR- and CMV-TCR-transduced T cells were expanded using at least 3 rounds of peptide stimulation followed by cell sorting, to obtain purified populations of CD8+ T cells expressing TCR only (CD8-TCR), CD4+ T cells expressing TCR only (CD4-TCR) and CD4+ T cells expressing TCR plus CD8 (CD4-TCR/CD8). The purity of T-cell populations and EBV-TCR expression is shown in panel (**C**), while cell purity and CMV-TCR expression is shown in panel (**D**). (**E and F**) Purified T-cell populations were simulated overnight with decreasing concentrations of the TCR-recognized peptide or a control peptide, followed by ELISA measurement of interferon γ (IFNγ, upper panels) and interleukin-2 (IL-2, lower panels) in the culture supernatant. Panel (**E**) shows the cytokine production of the indicated T-cell subpopulations expressing the EBV-TCR, while panel (**F**) shows the cytokine production by subpopulations expressing the CMV-TCR. Control stimulations were performed by using the highest concentration (1 μM) of control peptide (pNLV for EBV-TCR and pCLG for CMV-TCR).

MHC Class I-restricted CD4+ T cells mediate antigen-specific tumor protection in vivo. We used xenogeneic NOD/SCID mice to test whether human MHC Class I-restricted CD4+ T cells are able to protect against the growth of human tumor cells in vivo. Mice were subcutaneously challenged with 2×10^6 KA2 tumor cells expressing the relevant EBV or CMV antigen, followed by the intravenous transfer of purified CD8+ T cells expressing the relevant TCR or CD4+ T cells expressing the TCR alone or combined with CD8. The adoptive transfer of 2×10^6 CMV-specific CD8+ T cells into mice bearing pp65-expressing KA2-derived tumors resulted in tumor elimination in all mice by day 20 (**Fig. 6A**). The adoptive transfer of CD4+ T cells expressing the TCR only also resulted in tumor control, although detectable tumors persisted in all mice at day 20 and eradication was delayed to day 25. In contrast, CD4+ T cells expressing both the TCR and CD8 were capable of tumor eradication by

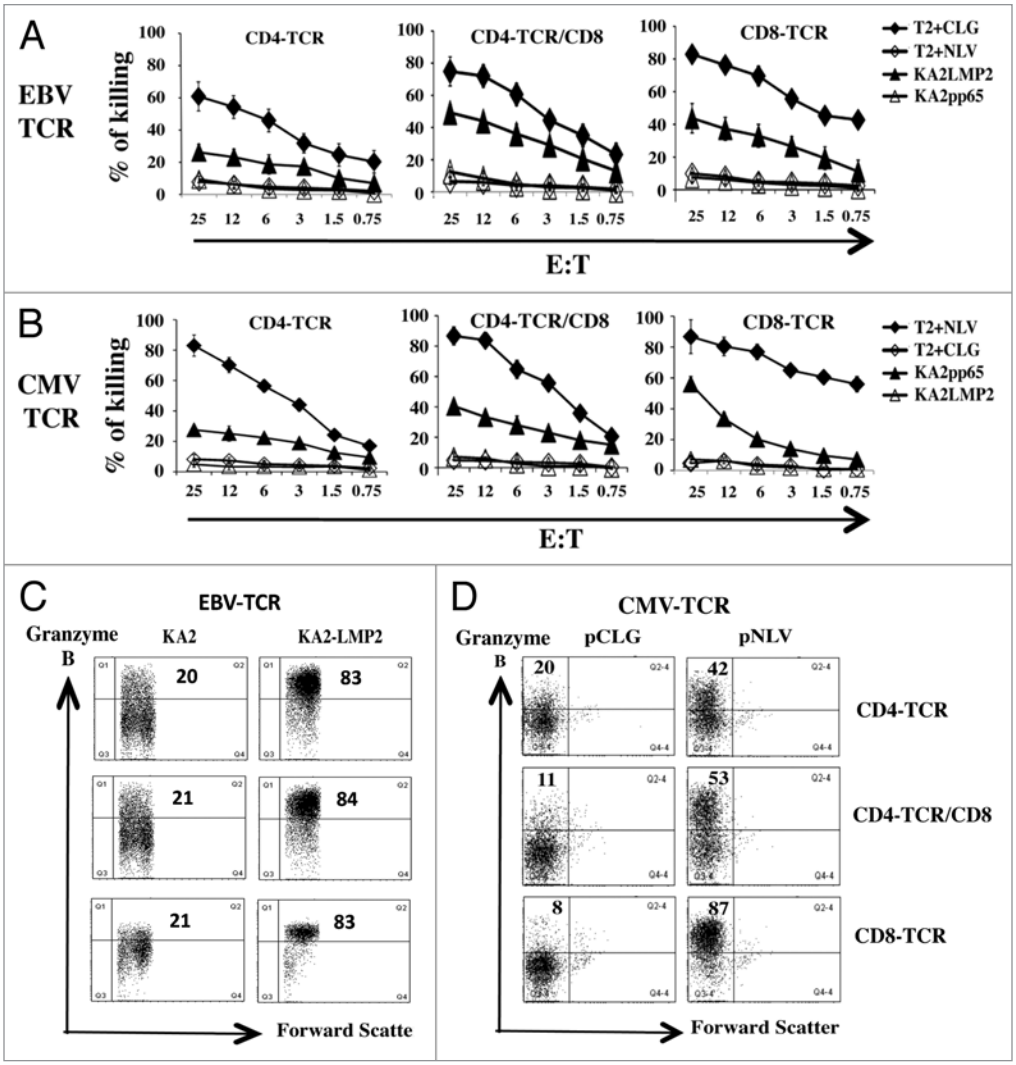

Figure 4. Antigen-specific killing and granzyme B production of TCR-engineered CD4+ T cells. (**A and B**) Cytotoxicity of EBV-TCR- (**A**) and CMV-TCR- (**B**) engineered CD4+ (CD4-TCR), CD8+ (CD8-TCR) and TCR-CD8αβ-engineered CD4+ (CD4-TCR/CD8) T cells. The purity of EBV-TCR- and CMV-TCR-engineered CD4+ T cells was 99%, and the purity of EBV-TCR-CD8αβ and CMV-TCR-CD8αβ engineered CD4+ T cells was 98%. A standard 4-h ^{51}Cr release assays was performed, and the percentage killing of target cells at different effector:target (E:T) ratios is shown. Data are representative of three independent experiments. (**C and D**) Intracellular granzyme B staining of EBV-TCR- (**C**) and CMV-TCR- (**D**) engineered CD4+ (CD4-TCR), CD8+ (CD8-TCR) and TCR-CD8αβ engineered CD4+ (CD4-TCR/CD8) T cells. EBV-TCR engineered T cells were stimulated overnight with KA2 tumor cells expressing or not the LMP2 antigen as indicated, while CMV-TCR-engineered T cells were stimulated with pCLG (control) or pNLV (specific) peptide-loaded T2 cells. The numbers in quadrants refer to the percentage of granzyme B-producing T cells. Data are representative of three independent experiments.

day 20. Control mice treated with transduced T cells expressing an irrelevant TCR (in this case the EBV-targeting TCR) developed progressively growing tumors, indicating that only CMV-TCR-engineered T cells provided protection against CMV antigen-expressing tumors. While control animals were sacrificed at day 25, due to large tumor burden, mice receiving CMV-TCR-engineered T cells remained tumor free until day 40, when the experiment was ended. **Figure 6B** compares the tumor burden in the 3 groups of mice receiving CMV-specific T-cell therapy, demonstrating that tumor control by CD4+ T cells expressing the TCR only was less effective than that by CD8+ T cells or by CD4+ T cells expressing both the TCR and CD8.

In the next series of experiments, mice were challenged with 2×10^6 KA2 tumor cells expressing the EBV LMP2 antigen, followed by the adoptive transfer of 2×10^6 purified CD8+ and CD4+ T cells expressing the EBV-targeting TCR. In this setting, CD4+ T cells expressing the TCR only or the TCR plus CD8 were unable to control tumor growth (**Fig. S3**). We then increased the T-cell dose to 3×10^6 cells per animal, which resulted in tumor rejection by all EBV-TCR-engineered T cell populations (**Fig. 7A**). However, while EBV-TCR-engineered CD8+ T cells as well as CD4+ T cells transduced with the TCR plus CD8 eliminated the tumors by day 20, this was delayed to day 25 in mice receiving with CD4+ T cells transduced with the TCR only. **Figure 7B** confirms that CD4+ T cells expressing the TCR only were less effective in controlling tumor growth than CD8+ T cells or CD4+ T cells expressing both the TCR and CD8. Again, mice treated with T cells expressing a control TCR (in this setting,

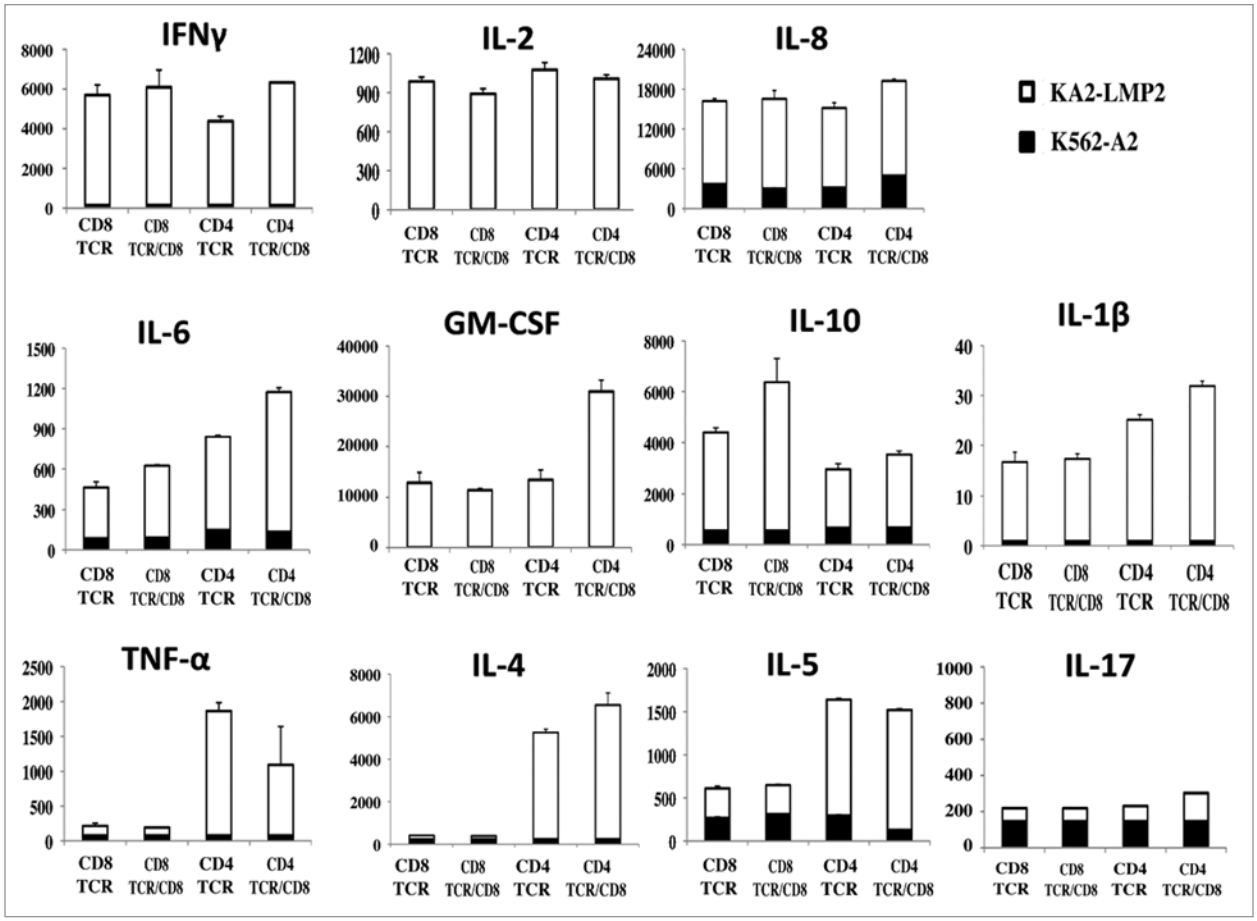

Figure 5. Comparison of the cytokine profile of EBV-TCR-engineered CD4+ and CD8+ T cells. Purified populations of CD8+ T cells (purity > 95%) and CD4+ T cells (purity > 99%) expressing TCR only or TCR plus CD8 were stimulated with control or LMP2-expressing (KA2-LMP2) KA2 cells. Following overnight stimulation, culture supernatants were analyzed on a luminex platform to determine the concentration of the 11 indicated cytokines. Open bars show cytokine production in response to KA2-LMP2 cells; black bars show control cytokine production in response to KA2 cells. Each panel shows the response of purified CD8+ and CD4+ T cells transduced with TCR only or with TCR plus CD8, as indicated. The luminex analysis was performed in two independent experiments with similar results.

the CMV-targeting TCR) exhibited progressive tumor growth, while the follow-up until day 40 of mice treated with EBV-TCR-engineered T cells showed lasting protection in all groups.

Altogether, these experiments clearly indicate that the transfer of TCR- plus CD8- coding genes improves the therapeutic efficacy of CD4+ T cells to a level that is similar to that achieved with TCR-transduced CD8+ T cells.

Discussion

A recent study in melanoma patients demonstrates that the adoptive transfer of CD4+ T cells is able to mediate tumor regression.[15] Similarly, a preclinical study in the B16 melanoma model indicates that the adoptive transfer of transgenic CD4+ T cells specific for the melanoma associated antigen Trp1 mediates more robust tumor protection than that of transgenic CD8+ T cells specific for the melanoma antigen gp100.[16] The reason why CD4+ T cells were more effective than CD8+ T cells may be related to differences in the effector mechanisms used by these T-cell subpopulations, or it simply may reflect the fact that different TCRs used by CD4+ and CD8+ T cells had different affinities for their respective MHC Class II- and MHC Class I-presented peptide antigens.

In order to distinguish between these possibilities we set out to generate CD4+ and CD8+ T cells that recognize the same peptide antigen with the same TCR affinity and the same functional avidity. Identical affinity was achieved by transfer of the same virus-specific TCR-coding genes into both CD4+ and CD8+ T cells. Since transferred TCRs were MHC Class I-restricted, we expected that the lack of the CD8 co-receptor would impair the functional avidity of engineered CD4+ T cells, which was confirmed in peptide titration experiments comparing CD4+ and CD8+ T cells expressing the same TCR (**Fig. 2**). These experiments revealed that the avidity of CD8+ T cells was approximately 10-fold greater than that of CD4+ T cells. The fact that such a difference in avidity was indeed due to the CD8 co-receptor was demonstrated by the transfer of the genes coding for CD8 α and β chains, which increased the avidity of CD4+ T cells to that observed in CD8+ T cells (**Fig. 3E and F; Fig. 4A and B; Figs. 6 and 7**). It is most likely that there are no truly CD8-independent TCR, but that the co-receptor has the ability to

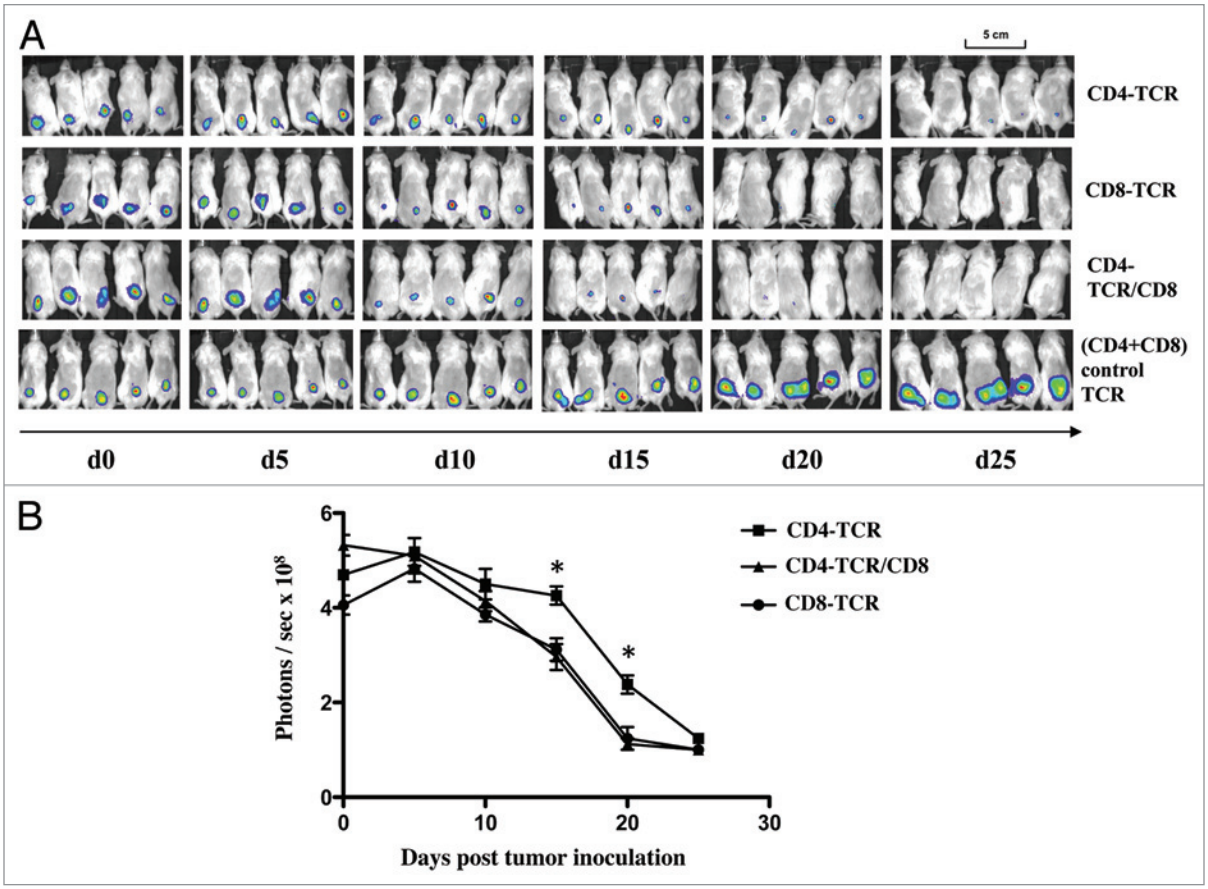

Figure 6. In vivo tumor clearance mediated by CMV-TCR-engineered T cells. (**A**) NOD/SCID mice were given 2.5 Gy irradiation at day -1, followed by subcutaneous inoculation of 2×10^6 KA2-pp65-Luc tumor cells at day 0 and intravenous injection of 2×10^6 T cells at day 1. Tumor growth was monitored in groups of 5 mice using bioluminescence imaging every 5 d, as indicated. The T-cell populations that were adoptively transferred into mice are indicated on the right hand side of the figure. The purity of the $CD4^+$ populations expressing TCR only (CD4-TCR) and TCR plus CD8 (CD4-TCR/CD8) was greater than 98%. Data are representative of two independent experiments. (**B**) Tumor burden (as measured by bioluminescence) was plotted against time. The plot shows the mean tumor burden (n = 5) for each of the 3 experimental groups. Error bars denote the standard error of the mean (SEM). The mean tumor burden of each of the experimental groups at day 15 and 20 was compared using a non-parametric (Mann-Whitney U) test. * $p < 0.025$ for the comparison of CD4-TCR with CD8-TCR and CD4-TCR/CD8 groups.

increase the functional avidity of T cells irrespective of TCR affinity.

The co-transfer of TCR- and CD8- coding genes into $CD4^+$ T cells also provides an experimental model to dissect in more detail the functional properties of the CD8 co-receptor. In this study, we transferred the genes encoding both CD8 α and β chains (**Fig. 3A**), while previous human studies only provided the CD8α chain, which triggered a change in cytokine production by the CD8α-expressing $CD4^+$ T cells.[30] In contrast, we found no change in cytokine production by $CD4^+$ T cells expressing the CD8 α and β chain (**Fig. 5**), suggesting that the human CD8α homodimer and the CD8α/β heterodimer have distinct functional properties, as was observed previously in murine CD8-coding gene transfer experiments.[31]

The in vivo experiments described here identify T-cell avidity as a key factor determining the control of tumor growth by MHC Class I-restricted $CD4^+$ T cells. Since the TCR plus CD8-engineered high avidity $CD4^+$ T cells retained the cytokine production profile of the low avidity $CD4^+$ T cells expressing the TCR only, the increased in vivo tumor protection potency appeared to be linked to the increased T-cell avidity. However, we also found that T-cell avidity is not the only indicator of tumor protection. In the setting of the EBV-targeting TCR, we noted that $CD8^+$ T cells are protective at a lower cell dose than TCR plus CD8-engineered $CD4^+$ T cells, although they both display the same functional avidity. At present, we do not know which effector mechanism accounts for the improved tumor protection by $CD8^+$ T cells expressing the EBV-targeting TCR. Interestingly, a recent study with an HLA-A2-restricted tyrosinase-specific TCR revealed similar protection against melanoma when TCR-engineered $CD4^+$ or $CD8^+$ T cells are transferred into mice conditioned by irradiation and treated with high dose IL-2.[32] Hence, it is possible that conditioning and high dose IL-2 are able to override differences in avidity and effector function between adoptively transferred $CD4^+$ or $CD8^+$ T cells.

In vitro affinity maturation, using TCR display in the yeast or phage system, provides an elegant strategy to improve the function of MHC Class I-restricted TCRs in $CD4^+$ T cells.[33,34]

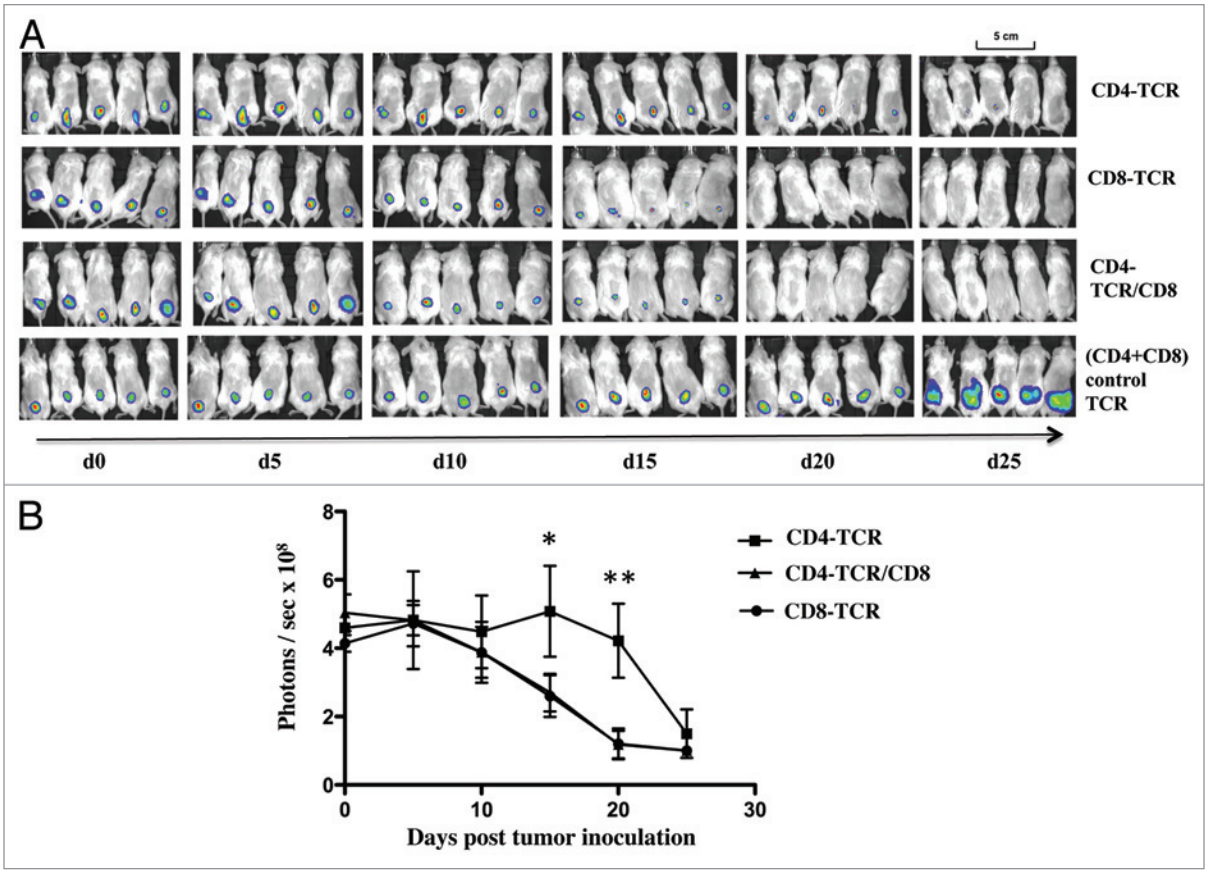

Figure 7. In vivo tumor clearance mediated by EBV-TCR engineered T cells. (**A**) NOD/SCID mice were given 2.5 Gy irradiation at day -1, followed by subcutaneous inoculation of 2×10^6 KA2-LMP2-Luc tumor cells at day 0 and intravenous injection of 3×10^6 T cells at day 1. Tumor growth was monitored in groups of 5 mice using bioluminescence imaging every 5 d as indicated. The T-cell populations that were adoptively transferred into mice are indicated on the right hand side of the figure. The purity of the CD4+ populations was 99% for the "TCR only" group (CD4-TCR), and 98% for "TCR plus CD8" group (CD4-TCR/CD8). Data are representative of two independent experiments. (**B**) Tumor burden (as measured by bioluminescence) was plotted against time. The plot shows the mean tumor burden (n = 5) for each of the 3 experimental groups. Error bars denote the standard error of the mean (SEM). The mean tumor burden of each of the experimental groups at day 15 and 20 was compared using a non-parametric (Mann-Whitney U) test. *$p < 0.025$, **$p < 0.001$ for the comparison of CD4-TCR with CD8-TCR and CD4-TCR/CD8 groups.

Experiments with the murine 2C TCR indicate that affinity maturation improves antigen-specific cytokine production in CD8⁻ hybridoma cells, while the same TCR lost antigen-specificity when analyzed in CD8⁺ hybridoma cells.[35] The analysis of affinity-matured versions of the human IG4 TCR specific for an HLA-A2-presented NY-ESO-1 peptide yielded similar results. Enhancing TCR affinity by 1000-fold resulted in the retention of antigen-specificity by CD4⁺ T cells, but also in non-specific cross-reactivity when the affinity-matured TCR was analyzed in human CD8⁺ T cells.[36] More modest affinity improvement of the IG4 TCR (10–20-fold K_D improvement) resulted in the retention of antigen-specificity in both CD8⁺ and CD4⁺ T cells.[37] These observations highlight the difficulties of using TCR affinity maturation to obtain CD4⁺ and CD8⁺ T cells displaying identical specificity and similar avidity for the relevant peptide antigen, a goal that is more easily achieved by the co-transfer of TCR- and CD8-coding genes. While our study has focused on optimizing the specificity and functional avidity of MHC I-restricted human CD4⁺ T cells, further studies are required to investigate optimal combination therapy with reduced doses of CD4⁺ and CD8⁺ T cells. These studies will reveal whether MHC Class I-restricted CD4⁺ T cells retain "classical" helper functions and improve the expansion and memory development of adoptively transferred CD8⁺ T cells. Ideally, these experiments need to be done with TCR-transduced murine T cells in HLA-A2 transgenic mice, which can overcome one of the limitations of the xenogeneic NOD/SCID model, namely the poor long-term persistence and memory formation of adoptively transferred human T cells in the murine host.

The EBV-specific TCR used here provides new therapy options for the treatment of lymphoma and nasopharygeal carcinoma, while the CMV-targeting TCR may be effective against medulloblastoma, or in controlling CMV reactivation/disease in transplant recipients. The incorporation of the CD8 α and β chain-coding genes into the next generation TCR gene therapy vectors should provide an opportunity to optimally recruit both CD4⁺ and CD8⁺ T cells to enhance tumor protection by engineered T cells.

Materials and Methods

Construction of retroviral EBV-TCR and CMV-TCR vectors. EBV-specific TCR variable regions of Vα14 and Vβ6 were amplified by high fidelity PCR from an LMP2-specific cytotoxic T lymphocyte clone[22] and linked with murine constant domains via *Not*I at the 5' end and *Sac*II at the 3' end for the α chain, and *Nco*I at the 5' end and *Xho*I at the 3' end for the β chain, in a pGA4 vector in which the α and β chains were linked via a p2A sequence as described previously.[38] The whole human-murine hybridized α and β chains were then transferred into the pMP71 retroviral vector through *Not*I at the 5' end, and *EcoR*I at the 3' end. An extra inter-chain disulphide bond between the murine constant domains α Cys48 and β Cys57 was introduced via site-directed mutagenesis as described previously.[28] We named this construct as EBV-TCR (**Fig. S1A**). The CMV-specific T cell receptor variable region sequences (Vα18 and Vβ13) were extracted from the public data set of published TCRs specific for CMV.[23] The TCR variable gene sequences were synthesized and linked with murine constant domains in a pGA4 vector using the same strategy as outlined above. The whole human-murine TCR α and β chains were then transferred into pMP71 retroviral vector through *Not*I at the 5' end, and *EcoR*I at the 3' end. We named this construct CMV-TCR (**Fig. S1A**). To generate the EBV-TCR or CMV-TCR linked with CD8 genes, the genes coding for human CD8 α and β chains were amplified from a cDNA library of human peripheral blood mononuclear cells (PBMCs), and then linked with EBV-TCR or CMV-TCR as TCRα-p2A-TCRβ-T2A-CD8α-E2A-CD8β (**Fig. 4A**).

Transduction of retroviral TCR-coding constructs into Jurkat and human PBMCs. Retroviral transduction of TCR-coding genes into human PBMCs was performed as described previously.[11] The Jurkat-76 cell line, which is a cloned human T-cell leukemia line deficient for endogenous TCR expression,[39] was transduced in the same way as for PBMCs but without the need for activation. Forty-eight hours after transduction, expression of the TCR or CD8 transgenes was analyzed by flow cytometry using anti-mCβ-APC, anti-CD8-FITC, EBV-pentamer-PE or CMV-tetramer-PE on a LSR II flow cytometer (BD Biosciences), and FACS data were analyzed using FACSDiva software.

Intracellular cytokine staining assays. This assay was performed in 96-well round-bottom plates. 2×10^5 TCR-engineered T cells were incubated with 2×10^5 tumor cell lines K562-A2 (KA2), KA2-LMP2 or KA2-pp65 as indicated in the figure legends. For peptide titration experiments, T2 cells were coated with a specific peptide (pCLG: CLG GLL TMV for EBV-specific T cells and pNLV: NLV PMV ATV for CMV-specific T cells) at serial 10-fold dilutions (as indicated in the figures) in 200 μL of culture medium containing brefeldin A (Sigma-Aldrich) at 1 μg/mL. After an incubation period of 18 h at 37°C with 5% CO_2, cells were first stained for surface CD4-APC-cy7 and CD8-APC then fixed, permeabilized and stained for intracellular IFNγ-FITC, IL-2-PE. For TNFα or granzyme B staining, TNFα-APC or granzymeB-APC reagents were used in separate experiment (in this setting, CD8-APC was replaced by CD8-PE) and staining was performed using the Fix and Perm kit (Invitrogen) according to the manufacturer's instructions. Samples were acquired on a LSR II flow cytometer and the data were analyzed using FACSDiva software (BD Biosciences).

Cytokine detection assay by ELISA. 5×10^4 purified T cells from EBV-TCR- engineered T cells were co-cultured with 5×10^4 specific (pCLG) or control (pNLV) peptide-coated KA2 cells, and purified T-cell populations from CMV-TCR engineered T cells were co-cultured with specific (pNLV) or control (pCLG) peptide-coated T2 cells in a 96-well plate as described above but without brefeldin A. Eighteen hours later, supernatants were collected and then used for the analysis of secreted IFNγ and IL-2 with a human enzyme linked immunosorbent assay (ELISA) kit (BD Biosciences), according to the manufacturer's instructions. Some of the collected supernatants were also used for multiple cytokine analysis using the luminex technology (FIDIS™ ALBIA, Biomedical Diagnostics) according to the manufacturer's instructions. Data were analyzed by means of the Microsoft Excel software (Redmont).

Expansion of TCR-transduced T cells. Freshly transduced bulk T cells were either used directly in functional assays or expanded by antigen specific stimulation as described previously.[11]

CTL assays. Cytotoxicity assays were performed as described.[11] Briefly, 10^6 T2 cells were incubated at 37°C for 1 h in 200 μL assay medium (RPMI 1640 medium containing 5% heat inactivated fetal calf serum) with 100 μM synthetic peptides (pNLV or pCLG). Tumor cells or peptide-coated T2 cells were then labeled with ^{51}Cr for l hour, washed and added to a serial 2-fold dilutions of effector cells in round-bottom, 96-well plates to obtain a total volume of 200 μL/well. Assay plates were incubated at 37°C, 5% CO_2, and after 4 h, 50 μL aliquots of supernatants was harvested, diluted with 150 μL of scintillation fluid and counted using a Wallac 1450 Microbeta Plus counter. The specific killing was calculated by the equation (experimental ^{51}Cr-release – spontaneous ^{51}Cr-release) / (maximum ^{51}Cr-release – spontaneous ^{51}Cr-release) × 100%.

In vivo tumor protection experiments. The in vivo animal experiments were performed following the University College London and UK Home Office guidelines for the care and use of laboratory animals. To generate a tumor cell line that could be visualized for the in vivo tumor monitoring, we transduced the leukemia cell line KA2[38] with the EBV antigen LMP2 or CMV antigen pp65 and a luciferase retroviral vector, followed by limiting dilution to clone out the tumor cells expressing either tumor antigen (LMP2 or pp65) plus luciferase. For tumor protection, immunodeficient NOD/SCID mice were given 2.5 Gy irradiation on day -1. On day 0, each mouse was subcutaneously inoculated with 2×10^6 tumor cells (KA2-LMP2-Luc cells for the EBV-specific T cells, and KA2-pp65-Luc for the CMV-specific T cells). On day 1, mice were randomly divided into different groups. In the treatment groups of the KA2-LMP2-Luc tumor, each mouse was intravenously injected with purified EBV-specific T cells as indicated in the figures. The control mice were given unpurified ($CD4^+$ plus $CD8^+$) CMV-specific T cells. In the treatment groups of the KA2-pp65-Luc tumor, each mouse was intravenously injected with purified CMV-specific T cells as indicated on the figures. The control mice were given unpurified

(CD4+ plus CD8+) EBV-specific T cells. Mice were maintained on irradiated food and water, and monitored for tumor growth by measuring both the volume and luciferase signal. The mice were sacrificed when the tumors in the control group grew to a maximal size as stipulated by Home Office regulations.

Statistical analysis. p values were calculated using a non-parametric (Mann Whitney U) test of statistical significance. These analyses were performed using Prism 5.0 (GraphPad) software.

Disclosure of Potential Conflicts of interest

H.J.S. is a consultant for Cell Medica.

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Supplemental Material

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/oncoimmunology/article/22590

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