# CD8$\alpha$ Coreceptor to Improve TCR Gene Transfer to Treat Melanoma: Down-Regulation of Tumor-Specific Production of IL-4, IL-5, and IL-10

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**Therapeutic success of TCR gene transfer to treat tumors depends on the ability of redirected T cells to become activated upon tumor recognition in vivo. Help provided by tumor-specific Th1 cells is reported to relieve T cells from an anergized state and to induce tumor regression. We recently demonstrated the ability to generate melanoma-specific Th1 cells by genetic introduction of both a CD8-dependent TCR and the CD8$\alpha$ coreceptor into CD4$^+$ T cells. In this study, we analyzed a TCR that binds Ag independently of CD8, a property generally preferred to induce tumor-specific T cell responses, and addressed the contribution of CD8$\alpha$ following introduction into TCR-transduced CD4$^+$ T cells. To this end, primary human CD4$^+$ T cells were gene transferred with a high-avidity TCR, and were shown not only to bind peptide/MHC class I, but also to effectively kill Ag-positive tumor cells in the absence of CD8$\alpha$. The introduction of CD8$\alpha$ up-regulates the tumor-specific production of TNF-$\alpha$ and IL-2 to some extent, but significantly down-regulates production of IL-4, IL-5, and IL-10 in CD4$^+$ T cells. The introduction of a mutated cysteine motif in CD8$\alpha$, which prevents its binding to LCK and linker for activation of T cells, did not adversely affect expression and T cell cytotoxicity, but counteracted the CD8$\alpha$-mediated down-regulation of IL-4 and IL-5, but not IL-10. In conclusion, CD8$\alpha$ down-regulates the production of major Th2-type cytokines, in part mediated by LCK and/or linker for activation of T cells, and may induce differentiation of tumor-specific Th1 cells, which makes this coreceptor an interesting candidate to improve the clinical potential of TCR gene transfer to treat cancer.** ***The Journal of Immunology,*** **2006, 177: 991–998.**

To initiate effective and persistent control of tumor growth by specific T cells, it is necessary that T cells continue to proliferate in vivo, repopulate the host, and enter the memory compartment. Adoptive transfer of Ag-specific T cells has recently shown therapeutic successes in the eradication of tumors in patients with metastatic melanoma (1, 2), and the treatment of EBV and CMV infections in stem cell and organ transplant patients (3–5). However, ex vivo expanded CD8$^+$ T cells do generally not persist beyond a few days after infusion into patients and are prevented from replicating or even functioning in vivo. The compromised antitumor efficacy of transferred CD8$^+$ T cells is thought to be a consequence of the tumor's inability to efficiently activate tumor-specific T cells due to significant immunosuppression and defective Th cell function (6, 7).

The administration of CD4$^+$ Th cells concurrently with CD8$^+$ T cells may prevent exhaustion of infused CD8$^+$ T cells (8, 9) and result in effective antitumor T cell responses (10). It has been well documented that CD4$^+$ T cells activate dendritic cells, thereby providing enhanced Ag presentation and costimulation via cross-linking of CD40, which leads to priming of Ag-specific CD8$^+$ CTL function (11). In fact, adoptive transfer of CD4$^+$ T cells results in de novo generation of Ag-specific CD8$^+$ T cells and concomitant tumor destruction (12). Furthermore, CD4$^+$ T cells activate CD8$^+$ T cells that are already present at the site of the tumor and enable them to traffic to and/or remain within the tumor (8), activate other immune cells, and induce an antitumor humoral response, and are a major source of IFN-$\gamma$, an effector cytokine with potent tumor-regressing activity via inhibition of tumor-induced angiogenesis (13) and the ability to activate tumor-infiltrating macrophages (14).

We explored the use of human CD4$^+$ T cells for antitumor therapy, and demonstrated that CD4$^+$ T cells can be redirected to MAGE-A1 Ag presented by HLA-A1 molecules on melanoma cells via TCR gene transfer, and that triggering of MAGE-A1/HLA-A1-specific immune responses, such as Ag-specific cytotoxicity and Th1-type cytokine production, required cointroduction of the CD8$\alpha$ coreceptor (15). A genetic strategy to induce Th1 differentiation of tumor-specific T cells such as genetic introduction of CD8$\alpha$ may prove relevant for TCR gene therapy because tumor-specific Th1 cells have been reported to induce potent and persistent tumor regression (12, 16).

Human CD8 is a heterodimeric protein consisting of an $\alpha$- and $\beta$-chain, each ~33 kDa, and is expressed on most thymocytes and about one-third of peripheral blood T cells, with a small portion of T cells expressing both CD8$\alpha\beta$ and CD8$\alpha\alpha$ dimers. Recent crystallographic data of human CD8$\alpha\alpha$ Ig-like domain and HLA-A2 show that one CD8$\alpha\alpha$ homodimer binds one MHC molecule in a manner similar to Ab-Ag binding (17), and that CD8 binding involves contact to the $\beta_2$-microglobulin, $\alpha_2$, and $\alpha_3$ domains of the peptide/MHC complex (the latter being the major binding domain). Importantly, structural data indicate that the binding of CD8$\alpha\alpha$ to peptide/MHC does not cause any significant changes to the TCR-binding platform of the peptide/MHC, and ensure that the

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specificity for the peptide/MHC is determined and dominated by the TCR. Interestingly, the binding of CD8 to peptide/MHC, when compared with the binding of TCR to peptide/MHC, is weak, but shows extremely fast kinetics (18, 19). Given the low binding affinity of CD8αα for peptide/MHC, it is remarkable that inhibition of this interaction, either via soluble CD8α or via membrane expressed and mutated MHC class I (to adversely affect the CD8:peptide/MHC interaction), clearly reduces T cell activation and target cell lysis by CTLs (20–22). To mediate T cell activation, CD8 gets recruited to the peptide/MHC:TCR complex, which enables the CD8α-chain-associated LCK to phosphorylate CD3ζ. Phosphorylation of CD3ζ is followed by (recruitment and) phosphorylation of ZAP-70 and linker for activation of T cells (LAT),[3] the latter of which also associates with CD8α to get in close proximity of the TCR, where it bridges proximal to more distal TCR signaling (23).

TCRαβ that bind Ag independently of CD8α's ability to structurally stabilize the TCR:peptide/MHC interaction are generally preferred to generate tumor-specific $CD4^+$ Th cells following gene transfer (24). In this study, we therefore addressed the contribution of CD8α to $CD4^+$ T cell responses following redirection with a CD8-independent TCRαβ. To this end, $CD4^+$ T cells were transduced with genes encoding a high-affinity gp100/HLA-A2-specific TCRαβ, which enabled them to bind the corresponding peptide/MHC class I ligand and exert gp100-specific cytotoxicity independent of CD8α. However, the introduction of CD8α significantly down-regulates the Ag-specific production of IL-4, IL-5, and IL-10 in T cells that, except for IL-10, depends on recruitment of LCK and/or LAT. The CD8α-induced bias of a tumor-specific T cell response toward a Th1-type T cell response may counteract the immune-suppressed status of tumor-bearing hosts and improve clinical TCR gene transfer to treat cancers.

## Materials and Methods

### *Cells and reagents*

PBL from healthy donors were obtained after approval by the Erasmus Medisch Centrum Medical Ethical Committee, and isolated and expanded, as described elsewhere (25). The melanoma cell lines BLM (HLA-$A2^{positive}$), BLM transfected with human gp100-encoding cDNA (BLMgp100), and FM3 (gp100/HLA-$A2^{positive}$, a gift from J. Zeuthen, Copenhagen, Denmark) were cultured, as described previously (26). The culture conditions for the melanoma cell line MZ2-MEL3.0 (MAGE-A1/HLA-$A1^{positive}$, a gift from P. Coulie, Brussels, Belgium) are described previously (27). The human embryonic kidney cell line 293T and the amphotropic packaging cell line Phoenix were grown in DMEM (Invitrogen Life Technologies) supplemented with 10% bovine calf serum (HyClone) and used to package retroviruses carrying RNA encoding TCRαβ and/or CD8α. K562, a chronic myelogenous leukemia cell line, was cultured in RPMI 1640 medium supplemented with 200 mM L-glutamine and 10% bovine calf serum. Contamination of cells with mycoplasm species was excluded by frequent PCR testing of cellular DNA with mycoplasm-specific primers (mycoplasm detection kit; American Type Culture Collection). The mAbs used in this study for cellular depletion, flow cytometric, and/or sorting purposes comprised PE-conjugated anti-TCR Vβ14 mAb; FITC-conjugated anti-CD4 mAb; and PE or nonconjugated anti-CD8α mAb (all obtained from BD Biosciences). Abs used for immune precipitation and immune detection included anti-CD8α mAb (UCHT-4, recognizing the extracellular part of human CD8α; Kordia Life Sciences) and anti-LCK mAb (3A5, recognizing aa 54-222 of human LCK; Santa Cruz Biotechnology). Anti-CD8α mAb (4H8; Sanquin Blood Supply Foundation) was also used as a blocking reagent in cytotoxicity assays. Other reagents used in this study were: RetroNectin (human fibronectin fragments CH-296; Takara Shuzo); gp100/HLA-A2 tetramer (ProImmune); gp100 peptide YLEPGPVTA (26); and PMA and PHA (Sigma-Aldrich).

### *Cloning of TCR and CD8α genes and transduction of human T lymphocytes*

The genes encoding gp100/HLA-A2-specific TCRαβ genes (i.e., gp100/A2 TCR) were derived from the high-avidity CTL-296 and inserted into the retroviral vector pBullet (26). The pBullet containing the human CD8α transgene (termed $CD8\alpha_t$) was described earlier (15). Using the pBullet:$CD8\alpha_t$ as template DNA, we generated a CD8α variant mutated at a conserved cysteine motif that is responsible for binding LCK (analogous to CD8α-MC-1/2 (28)) and LAT (23) (termed $CD8\alpha_t^{C215/7A}$). In short, two endogenous cysteines (aa 215 and 217, according to sequence reference number gi 4502688, with amino acid numbering starting at M) were targeted via mutagenesis (QuikChange Site-Directed Mutagenesis; Stratagene) and changed into alanines using the following primers (5′ to 3′): CD8α mutant forward, AGACGTGTTGCCAAAGCTCCCCGGCCT and CD8α mutant reverse, AGGCCGGGGAGCTTTGGCAACACGTCT (with introduced nucleotide mutations underlined). $CD8\alpha_t^{C215/7A}$ insert was checked by sequence analysis. The TCR, $CD8\alpha_t$, or $CD8\alpha_t^{C215/7A}$ genes were introduced into anti-CD3 mAb-activated primary human T cells, as described (26). Before gene transfer, T cells were enriched for $CD4^+$ T cells using a depleting anti-CD8α mAb and goat anti-mouse Ig-labeled magnetic beads (Dynal Biotech), according to the manufacturer's instructions. CD8α-negative T cells were used as recipients for TCR and CD8α genes, whereas nondepleted (bulk) T cells were used as recipients for TCR genes only.

### *Immune precipitation and Western blot*

T cell transductants ($2.5 \times 10^7$ cells) expressing gp100/A2 TCR and either $CD8\alpha_t$ or $CD8\alpha_t^{C215/7A}$ were lysed in 10 mM Tris-Cl (pH 7.6), 150 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40 (Sigma-Aldrich) for 20 min on ice in the presence of the enzyme inhibitors 50 μg/ml aprotinin, 50 μg/ml leupeptin, 0.2 mM PMSF, 1 mM $NaVO_3$, and 0.5 mM NaF (all obtained from Sigma-Aldrich). Cleared lysate was immune precipitated with anti-CD8α mAb (UCHT-4; 1 μg/ml) overnight at 4°C, followed by protein G-Sepharose coated with rabbit anti-mouse IgG for 1 h at 4°C. The immune precipitates were washed, loaded, and run on a 12% Tris-HCl gel (Bio-Rad), and transferred onto polyvinylidene difluoride membranes (Bio-Rad) under semidry conditions. The membranes were subsequently used for immune detection with anti-LCK mAb (3A5; 2 μg/ml) for 3 h at room temperature, followed by peroxidase-labeled sheep anti-mouse IgG (1:2500; Amersham Biosciences) for 30 min at room temperature and developed via chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce).

### *Flow cytometry of gene-modified T lymphocytes*

TCR-transduced T cells were analyzed for TCR and CD8α expression by flow cytometry using anti-TCR Vβ14, the gp100/HLA-A2 tetramer, and/or CD8α mAb. Viability of T cell populations was assessed by trypan blue exclusion, and only those with a viability >90% were immunostained for flow cytometric analysis. For immunostaining, $0.1–0.5 \times 10^6$ transduced T cells were washed with ice-cold PBS containing 0.5% BSA and 0.02% EDTA, and incubated with mAbs or tetramers on ice for 30 min. Upon completion of the immunostainings, cells were washed again, fixed (with 1% paraformaldehyde), and analyzed on a Cytomics FC-500 flow cytometer (Beckman Coulter). The dot plots or histograms represent viable T cells gated on forward and sideward light scatter signals. Sorting was performed on a FACSVantage (BD Biosciences) using peptide/MHC tetramers and/or CD8α mAb. Sorted T cells were validated for transgene expression(s) (>90%) and expanded in feeder plates before their testing.

### *Cytotoxicity assay*

Cytotoxic activity of T cell transductants was routinely measured in 6-h $^{51}Cr$ release assays, essentially as described (26). BLM target cells were loaded with gp100 peptide (10 μM final) for 45 min at 37°C/5% $CO_2$ before cocultivation with effector T cells. Cytotoxicity assays were performed with various E:T ratios, and in the presence of K562 cold target cells at a 30:1 ratio over $^{51}Cr$-labeled target cells. Some cytotoxicity assays were performed in the presence of blocking anti-CD8α mAb (10 μg/ml), added to the effector T cells 30 min before cocultivation with target cells. Percentage of specific cytolysis, i.e., specific $^{51}Cr$ release, was calculated as described previously (29).

### *Cytokine production*

To quantify the production of Th1- and Th2-type cytokines by TCR and/or CD8α-transduced T cells after Ag-specific stimulation, $6 \times 10^4$ T cells were cultured in the presence of $2 \times 10^4$ tumor cells for 18 h. As a positive

[3] Abbreviation used in this paper: LAT, linker for activation of T cell.

control, T cell transductants were stimulated with PHA and PMA. Supernatants were harvested, and levels of IL-2, IL-4, IL-5, IL-10, IFN-$\gamma$, and TNF-$\alpha$ were determined via Cytokine Bead Array (Th1/Th2 CBA kit; BD Biosciences), according to the manufacturer' s instructions. This flow cytometry-based technique allows the quantification of multiple cytokines simultaneously.

## Results

*The gp100/A2 TCR is able to bind peptide/MHC and mediate cytotoxicity independent of CD8$\alpha$*

To analyze the function of CD8$\alpha$ in antitumor T cell responses, not related to its ability to stabilize TCR-mediated binding of peptide/MHC class I, we made use of a gp100/A2 TCR that enables T cells to bind peptide/MHC class I in the absence of CD8$\alpha$ (i.e., a CD8-independent TCR). CD8 and $CD4^+$ gp100/A2 TCR transductants were made, as well as $CD4^+$ gp100/A2 TCR transductants with a cointroduced CD8$\alpha$ transgene (termed $CD8\alpha_t$). Fig. 1*a* shows high CD8$\alpha$ expression in both T cell populations expressing either endogenous CD8$\alpha$ or $CD8\alpha_t$, but no expression of CD8$\alpha$ in CD4-only T cells (*upper panels*). Fig. 1*b* demonstrates that gp100/A2 TCR-mediated binding of the relevant peptide/MHC ligand is independent of CD8$\alpha$. Next, the gp100-specific cytotoxic activity of these TCR and/or CD8$\alpha$ transductants was analyzed (Fig. 2). $CD4^+$ T cells transduced with gp100/A2 TCR lysed Ag-positive melanoma cells, but did not lyse melanoma cells lacking the relevant Ag. The cointroduction of CD8$\alpha$ did only slightly increase the cytolytic activity of gp100/A2 TCR transductants. Preincubation of $CD8^+$ T cells with anti-CD8$\alpha$ mAb did neither affect the binding of gp100/A2 tetramers (data not shown) nor the gp100-specific cytotoxicity (Fig. 2).

*CD8$\alpha$ inhibits production of IL-4, IL-5, and IL-10*

Next, the contribution of CD8$\alpha$ to TCR-mediated functions was followed up at the level of tumor-specific cytokine production. T

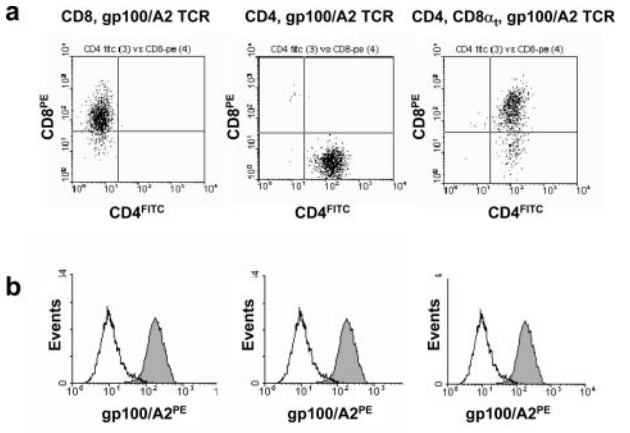


**FIGURE 1.** The gp100/A2-specific T cells do not require CD8$\alpha$ for binding peptide/MHC I ligand. CD8-positive and -negative fractions of primary human T cells were transduced with TCR genes derived from a gp100/A2-specific CTL-296 and, in case of CD8-negative T cells (i.e., $CD4^+$ T cells), also a $CD8\alpha_t$ gene. T cells were subsequently sorted into three populations with the use of PE-labeled gp100/A2 tetramer and FITC-labeled CD8$\alpha$ mAb: 1) $CD8^+$ T cells expressing the TCR$\alpha\beta$ transgenes; 2) $CD4^+$ T cells expressing the TCR$\alpha\beta$ transgenes; and 3) $CD4^+$ T cells expressing the TCR$\alpha\beta$ and $CD8\alpha_t$ transgenes. These three T cell populations were validated by flow cytometry for the expression of CD4 and CD8 (*a*) and binding with tetramers (*b*). See *Materials and Methods* for details on flow cytometry and sorting of T cells. Empty histograms represent staining with a nonrelated HLA-A2-binding tetramer. Data represent one of two donors with similar results.

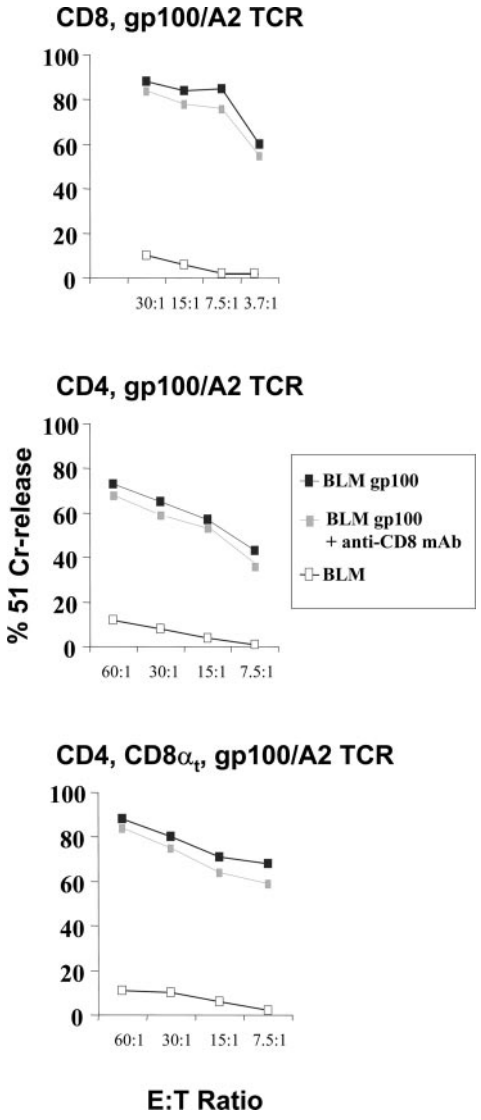


**FIGURE 2.** The gp100/A2-specific T cells do not require CD8$\alpha$ for tumor-specific cytotoxicity. The T cell populations shown in Fig. 1 were used as effector T cells in 6-h $^{51}Cr$ release assays. Target cells used were the HLA-A2-positive (but gp100-negative) melanoma cell line BLM and the gp100 transfectant BLMgp100. Cytotoxicity assays with the Ag-positive target cells were performed in the absence and presence of a blocking anti-CD8$\alpha$ mAb. See *Materials and Methods* for details on cytotoxicity assays. Data are presented as means from triplicate measurements with SEMs not exceeding 10%. Results were similar for two donors, each tested in two separate experiments.

cells expressing either endogenous CD8$\alpha$ or no CD8$\alpha$ (i.e., $CD4^+$ T cells) produce IL-4 and IL-5 in response to gp100-positive melanoma cells, especially gp100-transfected BLM cells, but also, albeit at lower levels, FM3 cells that natively express gp100, but not in response to gp100-negative melanoma cells (Fig. 3*a*). Surprisingly, the cointroduction of $CD8\alpha_t$ into $CD4^+$ TCR-transduced T cells almost completely abolished the gp100-specific production of both cytokines. The gp100-specific IL-10 production follows a different pattern. T cells expressing CD4 only, but not those expressing endogenous CD8$\alpha$, readily produce IL-10 in response to gp100-positive melanoma cells, which again becomes down-regulated in the copresence of $CD8\alpha_t$ (Fig. 3*a*). The CD8$\alpha$-mediated down-regulation of tumor-specific cytokine production by T cells appears specific for IL-4, IL-5, and IL-10. Ag-specific production of IFN-$\gamma$ only decreased slightly, whereas production of TNF-$\alpha$

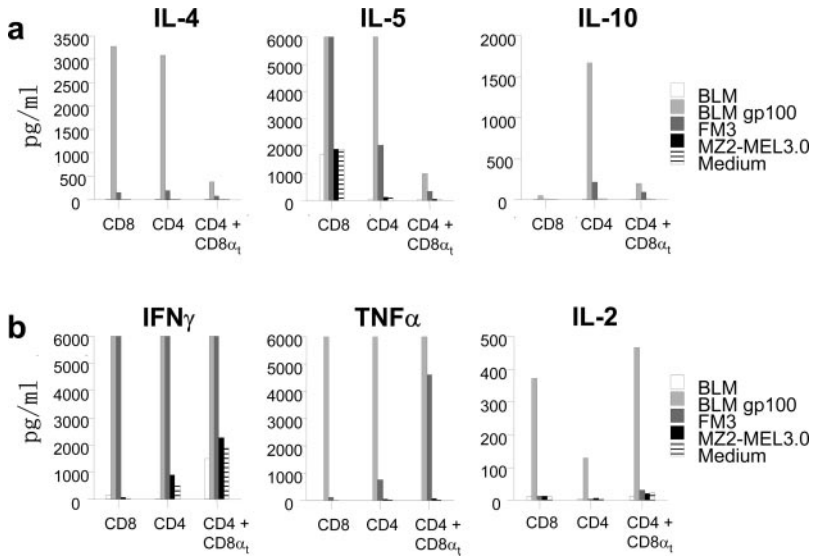


**FIGURE 3.** CD8α transgene expression in gp100/A2-specific CD4$^+$ T cells down-regulates gp100-specific production of IL-4, IL-5, and IL-10, but not IFN-γ, TNF-α, and IL-2. The T cell populations shown in Fig. 1 were used as effector T cells in cytokine production assays. Effector T cells were stimulated for 18 h at a 3:1 ratio using the following target cells: BLM, BLMgp100 (gp100/HLA-A2$^{positive}$), FM3, and (gp100/HLA-A2$^{neg}$) MZ2-MEL3.0. Negative and positive controls were medium and PMA/PHA stimulations, respectively. Cell-free supernatants were harvested and tested for the following cytokines: IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α via Cytometric Bead Array. *a*, The results for IL-4, IL-5, and IL-10 are given; *b*, the results for the other three cytokines are given. Cytokine levels exceeding 6000 pg/ml (shown as out of range) were as follows (responses to either BLMgp100 or FM3 cells separated by a slash and in ng/ml). *IFN-γ* − CD8: 89.6/17.0; CD4: 134.4/90.2; CD4 + CD8$\alpha_t$: 107.5/59.2. TNF-α − CD8: 6.3; CD4: 18.8; CD4 + CD8$\alpha_t$: 32.8. IL-5 − CD8: 24.7/6.5; CD4: 11.0. Data are presented as means from triplicate measurements with SEMs not exceeding 10%. Results were similar for two donors, each tested once.

and IL-2 increased because of cointroduction of CD8αt into T cells (see Fig. 3*b*).

*CD8α-mediated down-regulation of IL-4 and IL-5 production, but not IL-10, depends on cysteine motif*

To study the mechanism behind the CD8α-mediated decrease in tumor-specific production of the three Th2-type cytokines, we generated a CD8α mutant (termed CD8$\alpha_t^{C215/7A}$) in which two alanines substitute cysteines that are normally involved in the recruitment of intracellular LCK (see Fig. 4*a*) and LAT. CD8$\alpha_t^{C215/7A}$ was validated for its expression and inability to bind LCK in T cells (Fig. 4, *b* and *c*). Flow cytometric analysis of T cells cotransduced with gp100/A2 TCR and CD8$\alpha_t^{C215/7A}$ showed that the expression of the CD8α transgene was not affected by the cysteine to alanine mutations (compare Figs. 1*a* vs 4*b*). Coimmune precipitations using an anti-CD8α Ab confirmed that CD8$\alpha_t^{C215/7A}$ has a decreased ability to bind LCK and allowed us to use this mutant to analyze the role of CD8α-mediated signaling in Ag-specific T cell functions (Fig. 4*c*). The CD8$\alpha_t^{C215/7A}$ did not adversely affect the cytotoxic activity of T cells (Fig. 5). If anything, CD8$\alpha_t^{C215/7A}$ did slightly improve the cytotoxic ability of T cells transduced with gp100/A2 TCR (when compared with CD8$\alpha_t$). Next, we assessed the effect of CD8$\alpha_t^{C215/7A}$ on tumor-specific cytokine production by T cells. As put forward in Fig. 6*a*, the CD8α-mediated inhibition of tumor-specific IL-4 and IL-5 production depends on the ability of CD8α to recruit LCK and/or LAT because the introduction of CD8$\alpha_t^{C215/7A}$ does not inhibit the tumor-specific production of these cytokines. The involvement of CD8α-mediated signals to inhibit cytokine production appears to be restrictive in nature, because CD8α inhibits the tumor-specific production of IL-10 irrespective of a mutation in its cysteine motif (Fig. 6*a*). In addition, CD8$\alpha_t^{C215/7A}$ did not affect the tumor-specific production of IFN-γ, TNF-α, and IL-2 by T cells (Fig. 6*b*).

## Discussion

In the present work, we demonstrate that genetic introduction of CD8α into human CD4$^+$ T cells redirected with a high-avidity TCR contributes to tumor-specific Th1 responses. CD4$^+$ T cells transduced with gp100/A2-specific TCR bind peptide/MHC class I and kill gp100-positive tumor cells, neither being further enhanced by cointroduction of CD8$\alpha_t$ (Figs. 1 and 2). A study with another TCR, i.e., a MAGE-A1/HLA-A1-specific TCR, shows an absolute requirement of CD8α for both TCR-mediated binding of peptide/MHC class I and cytotoxicity (15). Gene transfer of the latter TCR results in cytotoxic responses toward peptide-loaded target cells that are 2 logs less sensitive compared with the gp100/A2 TCR used in the present study. We therefore believe that ligand-binding properties of the TCR dictate the CD8α dependence of T cell responsiveness. The presented results are in agreement with recent findings that high-affinity TCR:peptide/MHC interactions allow an accumulation of activation signals over time, resulting in CTL effector functions overriding the need for CD8 engagement (30). The observed killing of tumor cells by TCR-transduced CD4$^+$ T cells in short-term chromium release assays points to granzyme-mediated lysis as at least one of the mechanisms of killing. CD4$^+$ T cells have previously been reported to kill in an MHC class II-restricted and Ag-specific manner using primarily the Fas/Fas ligand and apoptosis-inducing pathways as well as, albeit it in general to a lesser extent, the exocytotic pathway (31). Interestingly, recent reports suggest that antitumor responses of TCR-transduced CD4$^+$ T cells may require MHC class II expression on target cells (32, 33). Cointroduction of WT1-specific TCR and *CD8α* genes appeared to rescue cytotoxicity, but not cytokine production by human Th1 cell toward naturally processed tumor Ag on MHC class II-negative leukemia cells (T. Nishimura, unpublished observations). In another study, although using murine CD4$^+$ T cells, the MHC class II dependence of *IFN-γ* production was shown to

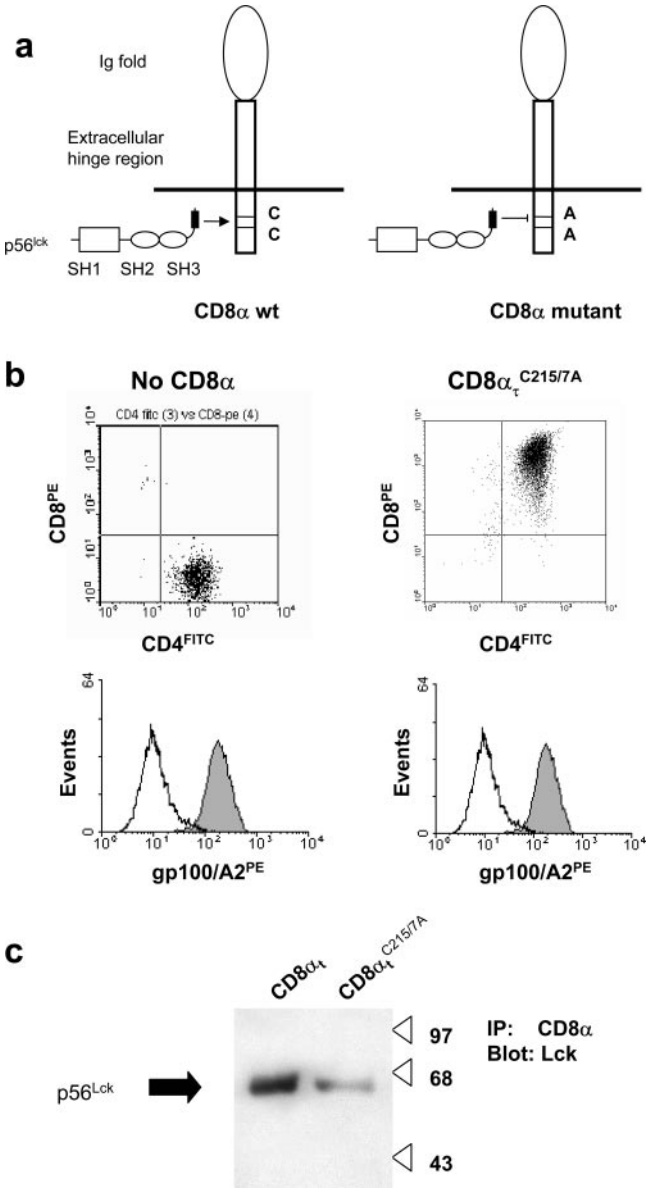


**FIGURE 4.** Generation and validation of CD8$\alpha$ mutant, termed CD8$\alpha_t^{C215/7A}$. CD8$\alpha_t$ was mutated at the cysteine motif (CKCP) by changing two cysteines into alanines, yielding CD8$\alpha_t^{C215/7A}$ (*a*). Mutation of the two cysteines (aa 215 and 217 according to sequence reference number gi 4502688, with amino acid numbering starting at M) prevents binding of $p56^{LCK}$ through its N-terminal aa 10–32 (indicated as ■) (28). Structural domains of both CD8$\alpha$ and $p56^{LCK}$ are indicated in *a*. Surface expression of this mutant was validated by flow cytometric analysis of $CD4^+$ human T cells either mock transduced or transduced with and sorted for both the gp100/A2 TCR and CD8$\alpha_t^{C215/7A}$ (*b*). *Upper panels*, Show expressions of CD4 and CD8, whereas the *lower panels* depict binding to tetramer. Expression profiles are similar to those using the CD8$\alpha_t$ (see Fig. 1). *c*, The decreased ability of CD8$\alpha_t^{C215/7A}$ to recruit LCK is demonstrated by coimmune precipitations. $CD4^+$ T cells expressing either CD8$\alpha_t$ or CD8$\alpha_t^{C215/7A}$, while growing at log phase under normal culture conditions, were lysed and used for CD8$\alpha$ immune precipitations (UCHT-4 mAb). Immune precipitates were loaded on a 12% Tris-HCl gel, blotted onto polyvinylidene difluoride membranes, and immune detected using anti-LCK mAb (3A5).

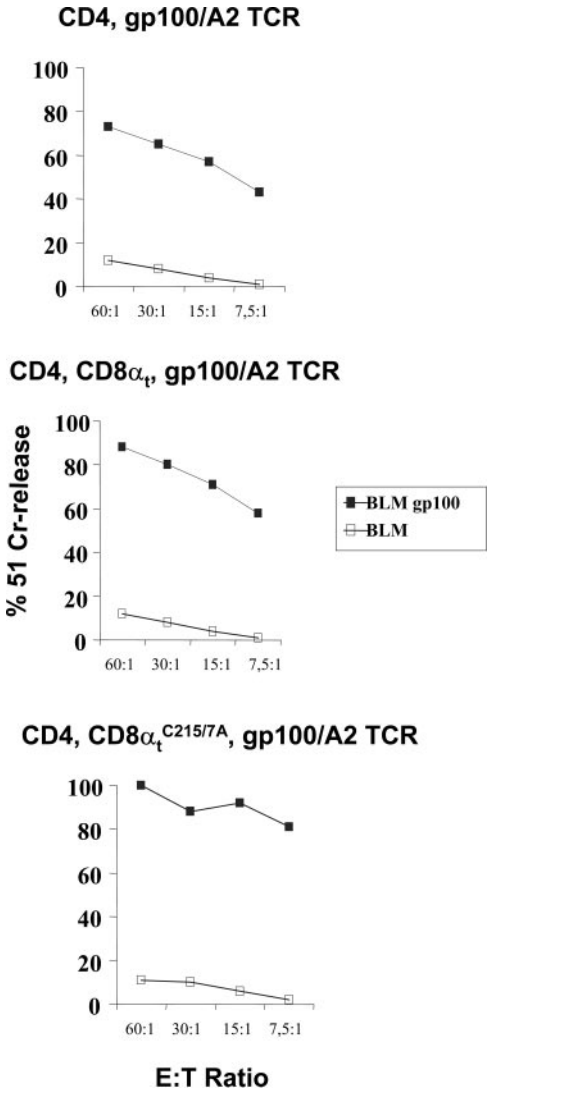


**FIGURE 5.** CD8$\alpha_t^{C215/7A}$ does not adversely affect tumor-specific T cell cytotoxicity. Three T cell populations expressing gp100/A2 TCR and either no CD8$\alpha$, CD8$\alpha_t$, or CD8$\alpha_t^{C215/7A}$ (see Figs. 1 and 4 for the corresponding flow cytometry data) were used as effector T cells in $^{51}Cr$ release assays. See legend to Fig. 2 for details on the use of target cells and assay conditions. Data are presented as means from triplicate measurements with SEMs not exceeding 10%. Results were similar for two donors, each tested in two separate experiments.

be relieved by cointroduction of *CD8α* (33). Our own data do not point to a significant contribution of MHC class II to the responsiveness of TCR-transduced $CD4^+$ T cells. First, target cells used in the present study were negative for MHC class II (data not shown), but did not prevent TCR-transduced $CD4^+$ T cells to both kill and produce cytokines in response to gp100 Ag in the absence of *CD8α*. Second, MAGE-A1-positive target cells (such as MZ2-MEL2.2 and 3.0) were clearly positive for MHC class II, and were only able to elicit both cytotoxicity and cytokine production by TCR-transduced $CD4^+$ T cells in the presence of *CD8α* (15). To address the contribution of CD4 signaling to *CD8α*-dependent T cell responsiveness more directly, experiments to block MHC class II in the context of MAGE-A1/HLA-A1-redirected $CD4^+$ T cells are currently ongoing in our laboratory.

In this study, $CD4^+$ T cells show CD8-independent tumor-specific production of both Th1- and Th2-type cytokines, possibly due to prominent TCR oligomerization and formation of stable immune synapses directed by the high-avidity gp100/A2 TCR. The genetic introduction of *CD8α* into $CD4^+$ T cells drastically downregulates the Ag-specific production of IL-4, IL-5, and IL-10 (Fig. 3), but not other Th2-type cytokines such as IL-6, IL-13, and IL-15

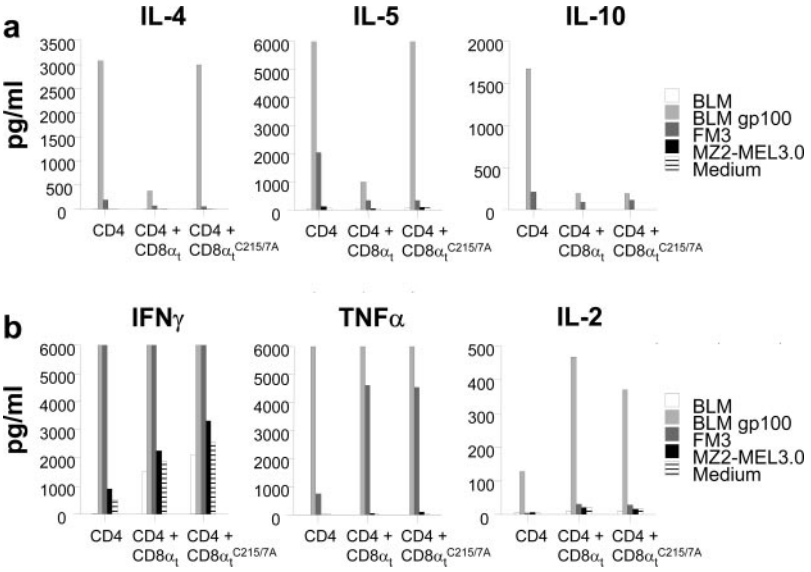


**FIGURE 6.** CD8α-mediated down-regulation of gp100-specific production of IL-4 and IL-5, but not IL-10, depends on the ability of CD8α to mediate intracellular signaling. The T cell populations shown in Fig. 5 were used in cytokine production assays. Conditions are as described in the legend to Fig. 3. Cell-free supernatants were harvested and tested for cytokines via Cytometric Bead Array. *a*, The results for IL-4, IL-5, and IL-10 are given; *b*, the results for the other three cytokines are given. Cytokine levels exceeding 6000 pg/ml (shown as out of range) were as follows (responses to either BLMgp100 or FM3 cells separated by a slash and in ng/ml). *IFN-γ* − CD4: 134.4/90.2; CD4 + $CD8\alpha_t$: 107.5/59.2; CD4 + $CD8\alpha_t^{C215/7A}$: 80.6/48.3. TNF-α − CD4 + $CD8\alpha_t^{C215/7A}$: 32.2. IL-5 − CD4 + $CD8\alpha_t^{C215/7A}$: 6.3. Data are presented as means from triplicate measurements with SEMs not exceeding 10%. Results were similar for two donors, each tested once.

(data not shown). The down-regulatory effects of CD8α may be typical for IL-4, IL-5, and IL-10, and may adversely affect a humoral and eosinophilic response, and is likely to promote a Th1-type response (by releasing a negative feedback loop via IL-10) (34). CD8α may facilitate, albeit via different mechanisms, tumor-specific Th1-type responses in $CD4^+$ T cells redirected with either a CD8-dependent TCR specific for MAGE-A1/HLA-A1 (up-regulation of Th1-type cytokine production (15)) or a CD8-independent TCR specific for gp100/A2 (down-regulation of the Th2-type cytokines IL-4, IL-5, and IL-10). The recent observation that human $CD4^+$ T cells retargeted with another CD8-independent TCR (i.e., p53/HLA-A2-specific TCR), but not a CD8-dependent TCR (i.e., murine double-minute 2 oncoprotein/HLA-A2-specific TCR), is also able to produce Th1-type cytokines (Th2-type cytokines were not tested) following Ag-specific stimulation (24), suggests that the Th1-inducing effect from introduced CD8α into TCR-modified $CD4^+$ T cells may not be restricted to certain TCRs.

CD8α containing a mutated LCK and LAT binding domain, termed $CD8\alpha_t^{C215/7A}$, enabled analysis of the CD8-mediated signaling with respect to down-regulation of IL-4, IL-5, and IL-10 production. The CD8α mutant expresses well, does not affect the cytotoxic activity of T cells, and shows reduced binding to LCK (binding to LAT was not tested) (Figs. 4 and 5). The introduced cysteine to alanine mutations in CD8α do, in contrast to CD4, not completely abolish its association with LCK, thereby confirming the initial biochemical characterization of this CD8α mutant (28). The mutational effect, however, is reported to drastically decrease CD8α-associated kinase activity (28). Genetic introduction of $CD8\alpha_t^{C215/7A}$ in our T cells does not adversely affect the tumor-specific production of IFN-γ, TNF-α, and IL-2, but points to a central involvement of LCK and/or LAT to CD8-mediated down-regulation of the tumor-specific production of IL-4 and IL-5, but not IL-10 (Fig. 6). In fact, both LCK and LAT have been reported to mediate signals leading to down-regulated production of Th2-type cytokines in $CD4^+$ T cells. First, LCK-deficient T cells or treatment of T cells with a kinase-dead LCK result in elevated production of Th2-type cytokines (35, 36). Second, compromised LCK may result in insufficient affinity maturation of LFA-1, and thereby lead to a Th2 phenotype (activated LFA-1 is normally able to inhibit STAT6-dependent up-regulation of GATA-3 and Th2-type cytokines; for review see Ref. 37). In this respect, it is particularly interesting that LFA-1-mediated adhesion is reported to be a critical element for tumor eradication by adoptive transfer of Th1, but not Th2 cells (38). Third, LCK is capable of tyrosine phosphorylating and activating Jaks as well as STAT factors (39, 40), and may either via the Jak-STAT pathway or other means affect the production of the transcription factors T-bet and GATA-3, resulting in a decreased production of IL-4 and IL-5. Fourth, members of the Tec family of protein kinases can be activated through phosphorylation by LCK and are subsequently able to regulate Th cell differentiation (41). For instance, IL-2 tyrosine kinase may skew Th cells toward a Th2 phenotype, possibly through interactions with LAT and activation of phospholipase C-γ1 (41). Finally, mutating one of LAT's tyrosines (Y136 in mice, equivalent to Y132 in humans) results in chronic production of large amounts of Th2-type cytokines (42). The Y136 mutation selectively eliminates binding of phospholipase C-γ1, thereby compromising a calcium flux, pointing to a negative effect from intracellular calcium on IL-4 and IL-5 production.

It is noteworthy that the effects of *CD8α*-mediated signaling on IL-4, IL-5, and IL-10 production appear not to hold true for endogenous *CD8α* (see Fig. 3). We postulate that the apparent differences between exogenous and endogenous *CD8α* are caused by differential routes of LCK and possibly LAT recruitment to the TCR complex and signaling pathways that may exist between $CD4^+$ and $CD8^+$ T cell subsets. In example, lipid rafts from $CD8^+$ T cells, but not $CD4^+$ T cells, already contain LCK before activation and do not polarize to the T cell:target cell contact site (43). In addition, *CD8β* (not present on TCR/CD8α-transduced $CD4^+$ T cells) has an intracellular palmitoylation signal that is reported to enable recruitment of TCR:CD8 complexes to lipid rafts (44), and enhances the *CD8α*-associated binding and activation of LCK (28) as well as LAT (23). Lastly, activation of components upstream of GATA3, such as STAT6, is controlled differently in CD4 vs $CD8^+$ T cells (45). The exact mechanisms downstream of CD8α-associated LCK (or LAT) that affect the Ag-specific production of IL-4 and IL-5 on the one hand and IL-10 in contrast are currently under investigation. In this respect, it should be noted that presented data reflect bulk cultures of T cells, and that one cannot exclude the possibility that at a single cell level responses may operate differently.

$CD4^+$ T cells producing Th1-type cytokines have been reported to display potent antitumor activities in T cell transfer studies (12,

38, 46, 47). For instance, OVA-specific murine $CD4^+$ T cells producing Th1-type cytokines, but not those producing Th2-type cytokines, eradicate (OVA-positive) tumors in T cell transfer studies by induction of OVA-specific $CD8^+$ T cells and maintenance of CTL memory (38). In addition, $CD4^+$ T cells retargeted with an Ab-based receptor and producing Th1-type cytokines display antitumor effects in xenotransplantation models using tumors positive for either human CEA or ErbB2 and synergize with $CD8^+$ T cells retargeted with the same receptor (46, 48). The introduction of local help to CTL by Th cells, preferably producing Th1-type cytokines, is considered crucial to overcome immunosuppression in tumor-bearing hosts. In this study, we demonstrate that genetic introduction of CD8$\alpha$ into $CD4^+$ T cells induces the generation of tumor-specific Th1 cells and may improve efficacy of TCR gene therapy to treat cancers. Successful development of this strategy also warrants studies into safety aspects. For instance, the risk of autoimmunity by activation of "ignorant" autoreactive T cells that are triggered by the introduced TCR may be affected by strategies that impact TCR signaling such as the one presented in this study. In our opinion, however, CD8$\alpha$ may not get recruited to MHC class II-restricted TCRs, making it unlikely that this coreceptor aids the endogenous TCR signaling of $CD4^+$ T cells. Another safety aspect is possible mispairing of TCR chains (i.e., formation of new *TCR*$\alpha\beta$ heterodimers comprising both introduced and endogenous TCR chains) that may again result in autoimmune reactions. To address TCR mispairing, our laboratory pioneered the development of various alternative formats for MHC-restricted receptors (49). Finally, insertional mutagenesis as a consequence of retroviral gene transfer may represent a serious safety issue, as it has already led to a third case of leukemia in the treatment of X-SCID with progenitor cells transduced with common $\gamma$-chain (50). Although insertional mutagenesis needs to be addressed in gene transfer studies using integrating viruses, it is important to state that, to date, in contrast to hemopoietic stem cells, there is no evidence of adverse effects of retroviral gene transfer into mature T cells.

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## Disclosures

The authors have no financial conflict of interest.

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