

CD8 α 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 α 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 α 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 α . The introduction of CD8 α up-regulates the tumor-specific production of TNF- α 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 α , 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 α -mediated down-regulation of IL-4 and IL-5, but not IL-10. In conclusion, CD8 α 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- γ , 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 coinfection of the CD8 α coreceptor (15). A genetic strategy to induce Th1 differentiation of tumor-specific T cells such as genetic introduction of CD8 α 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 α - and β -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 β_2 -microglobulin, α_2 , and α_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 CD8 α for peptide/MHC, it is remarkable that inhibition of this interaction, either via soluble CD8 α 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 CD8 α -chain-associated LCK to phosphorylate CD3 ζ . Phosphorylation of CD3 ζ 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 CD8 α to get in close proximity of the TCR, where it bridges proximal to more distal TCR signaling (23).

TCR $\alpha\beta$ that bind Ag independently of CD8 α '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 CD8 α to CD4⁺ T cell responses following redirection with a CD8-independent TCR $\alpha\beta$. To this end, CD4⁺ T cells were transduced with genes encoding a high-affinity gp100/HLA-A2-specific TCR $\alpha\beta$, which enabled them to bind the corresponding peptide/MHC class I ligand and exert gp100-specific cytotoxicity independent of CD8 α . However, the introduction of CD8 α 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 CD8 α -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 TCR $\alpha\beta$ and/or CD8 α . 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 mycoplasma species was excluded by frequent PCR testing of cellular DNA with mycoplasma-specific primers (mycoplasma 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 V β 14 mAb; FITC-conjugated anti-CD4 mAb; and PE or nonconjugated anti-CD8 α mAb (all obtained from BD Biosciences). Abs used for immune precipitation and immune detection included anti-CD8 α mAb (UCHT-4, recognizing the extracellular part of human CD8 α ; Kordia Life Sciences) and anti-LCK mAb (3A5, recognizing aa 54-222 of human LCK; Santa Cruz Biotechnology). Anti-CD8 α 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 YLEPGPVA (26); and PMA and PHA (Sigma-Aldrich).

Cloning of TCR and CD8 α genes and transduction of human T lymphocytes

The genes encoding gp100/HLA-A2-specific TCR $\alpha\beta$ 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 CD8 α transgene (termed CD8 α) was described earlier (15). Using the pBullet:CD8 α as template DNA, we generated a CD8 α variant mutated at a conserved cysteine motif that is responsible for binding LCK (analogous to CD8 α -MC-1/2 (28)) and LAT (23) (termed CD8 α ^{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'): CD8 α mutant forward, AGACGTGTTGCCAAAGCTCCCCGGCT and CD8 α mutant reverse, AGGCCGGGAGCTTTGGCAACACGTCT (with introduced nucleotide mutations underlined). CD8 α ^{C215/7A} insert was checked by sequence analysis. The TCR, CD8 α , or CD8 α ^{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-CD8 α mAb and goat anti-mouse Ig-labeled magnetic beads (DynaL Biotek), according to the manufacturer's instructions. CD8 α -negative T cells were used as recipients for TCR and CD8 α genes, whereas nondepleted (bulk) T cells were used as recipients for TCR genes only.

Immune precipitation and Western blot

T cell transductants (2.5×10^7 cells) expressing gp100/A2 TCR and either CD8 α or CD8 α ^{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 μ g/ml aprotinin, 50 μ g/ml leupeptin, 0.2 mM PMSF, 1 mM NaVO₃, and 0.5 mM NaF (all obtained from Sigma-Aldrich). Cleared lysate was immune precipitated with anti-CD8 α mAb (UCHT-4; 1 μ g/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 μ g/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 CD8 α expression by flow cytometry using anti-TCR V β 14, the gp100/HLA-A2 tetramer, and/or CD8 α 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, $0.1\text{--}0.5 \times 10^6$ 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 CD8 α mAb. Sorted T cells were validated for transgene expression (>90%) and expanded in feeder plates before their testing.

Cytotoxicity assay

Cytotoxic activity of T cell transductants was routinely measured in 6-h ⁵¹Cr release assays, essentially as described (26). BLM target cells were loaded with gp100 peptide (10 μ M final) for 45 min at 37°C/5% CO₂ 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 ⁵¹Cr-labeled target cells. Some cytotoxicity assays were performed in the presence of blocking anti-CD8 α mAb (10 μ g/ml), added to the effector T cells 30 min before cocultivation with target cells. Percentage of specific cytolysis, i.e., specific ⁵¹Cr release, was calculated as described previously (29).

Cytokine production

To quantify the production of Th1- and Th2-type cytokines by TCR and/or CD8 α -transduced T cells after Ag-specific stimulation, 6×10^4 T cells were cultured in the presence of 2×10^4 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- γ , and TNF- α 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 α

To analyze the function of CD8 α 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 α (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 α transgene (termed CD8 α_t). Fig. 1*a* shows high CD8 α expression in both T cell populations expressing either endogenous CD8 α or CD8 α_t , but no expression of CD8 α 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 α . Next, the gp100-specific cytotoxic activity of these TCR and/or CD8 α 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 α did only slightly increase the cytolytic activity of gp100/A2 TCR transductants. Preincubation of CD8⁺ T cells with anti-CD8 α mAb did neither affect the binding of gp100/A2 tetramers (data not shown) nor the gp100-specific cytotoxicity (Fig. 2).

CD8 α inhibits production of IL-4, IL-5, and IL-10

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

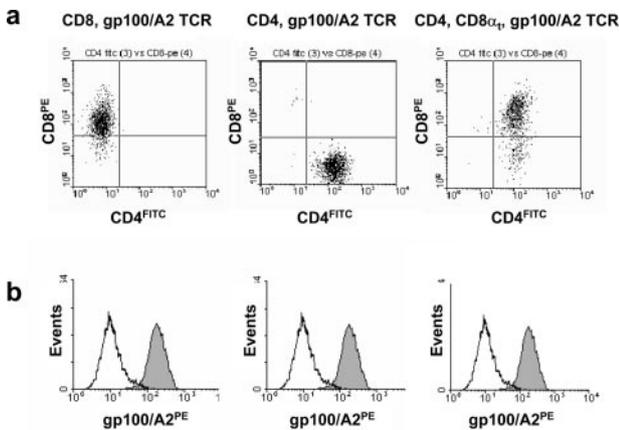


FIGURE 1. The gp100/A2-specific T cells do not require CD8 α for binding peptide/MHC I ligand. CD8-positive and -negative fractions of primary human T cells were transduced with TCR genes derived from a gp100/A2-specific CTL-296 and, in case of CD8-negative T cells (i.e., CD4⁺ T cells), also a CD8 α_t gene. T cells were subsequently sorted into three populations with the use of PE-labeled gp100/A2 tetramer and FITC-labeled CD8 α 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 α_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.

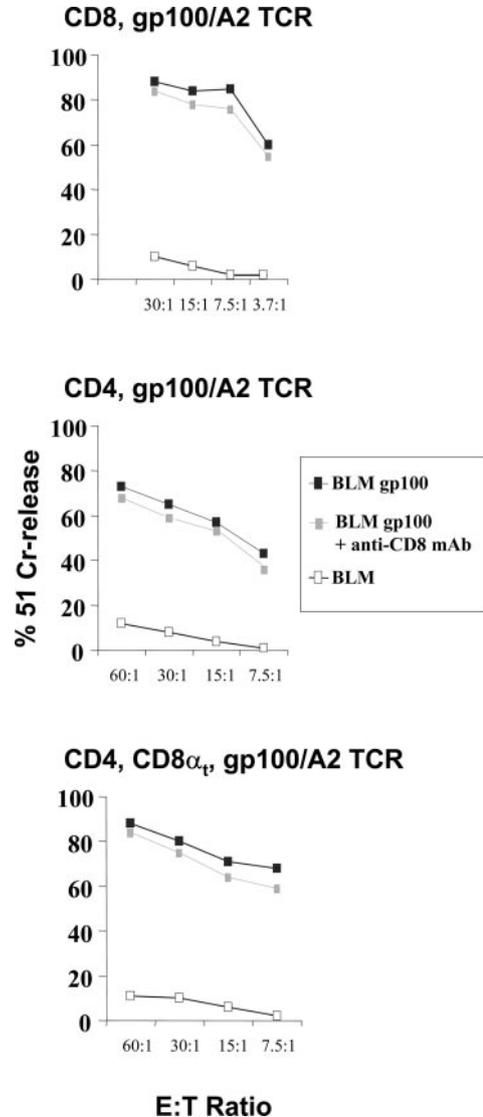


FIGURE 2. The gp100/A2-specific T cells do not require CD8 α for tumor-specific cytotoxicity. The T cell populations shown in Fig. 1 were used as effector T cells in 6-h ⁵¹Cr release assays. Target cells used were the HLA-A2-positive (but gp100-negative) melanoma cell line BLM and the gp100 transfectant BLMgp100. Cytotoxicity assays with the Ag-positive target cells were performed in the absence and presence of a blocking anti-CD8 α 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 α or no CD8 α (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 α_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 α , readily produce IL-10 in response to gp100-positive melanoma cells, which again becomes down-regulated in the copresence of CD8 α_t (Fig. 3*a*). The CD8 α -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- γ only decreased slightly, whereas production of TNF- α

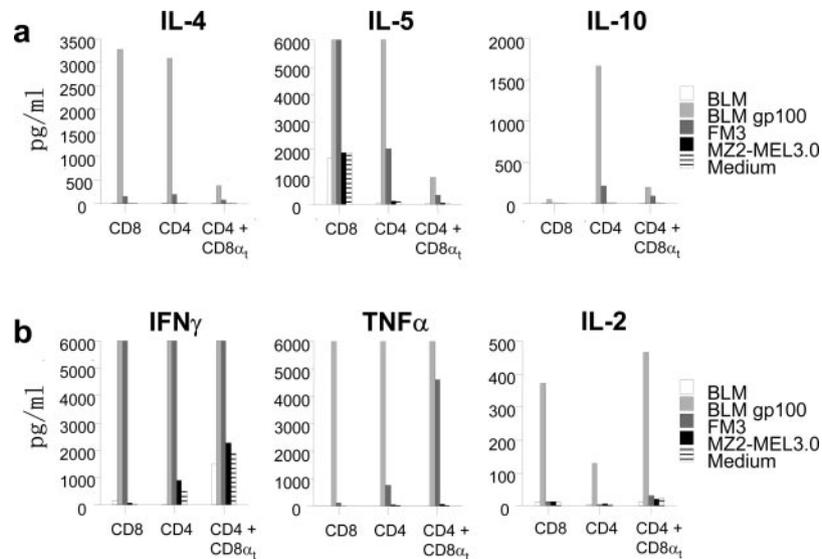


FIGURE 3. CD8 α transgene expression in gp100/A2-specific CD4⁺ T cells down-regulates gp100-specific production of IL-4, IL-5, and IL-10, but not IFN- γ , TNF- α , and IL-2. The T cell populations shown in Fig. 1 were used as effector T cells in cytokine production assays. Effector T cells were stimulated 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 α : 107.5/59.2. TNF- α – CD8: 6.3; CD4: 18.8; CD4 + CD8 α : 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 CD8 α into T cells (see Fig. 3*b*).

CD8 α -mediated down-regulation of IL-4 and IL-5 production, but not IL-10, depends on cysteine motif

To study the mechanism behind the CD8 α -mediated decrease in tumor-specific production of the three Th2-type cytokines, we generated a CD8 α mutant (termed CD8 α _{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 α _{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 α _{C215/7A} showed that the expression of the CD8 α transgene was not affected by the cysteine to alanine mutations (compare Figs. 1*a* vs 4*b*). Coimmune precipitations using an anti-CD8 α Ab confirmed that CD8 α _{C215/7A} has a decreased ability to bind LCK and allowed us to use this mutant to analyze the role of CD8 α -mediated signaling in Ag-specific T cell functions (Fig. 4*c*). The CD8 α _{C215/7A} did not adversely affect the cytotoxic activity of T cells (Fig. 5). If anything, CD8 α _{C215/7A} did slightly improve the cytotoxic ability of T cells transduced with gp100/A2 TCR (when compared with CD8 α). Next, we assessed the effect of CD8 α _{C215/7A} on tumor-specific cytokine production by T cells. As put forward in Fig. 6*a*, the CD8 α -mediated inhibition of tumor-specific IL-4 and IL-5 production depends on the ability of CD8 α to recruit LCK and/or LAT because the introduction of CD8 α _{C215/7A} does not inhibit the tumor-specific production of these cytokines. The involvement of CD8 α -mediated signals to inhibit cytokine production appears to be restrictive in nature, because CD8 α inhibits the tumor-specific production of IL-10 irrespective of a mutation in its cysteine motif (Fig. 6*a*). In addition, CD8 α _{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 CD8 α 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 α (Figs. 1 and 2). A study with another TCR, i.e., a MAGE-A1/HLA-A1-specific TCR, shows an absolute requirement of CD8 α 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 CD8 α 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 CD8 α 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

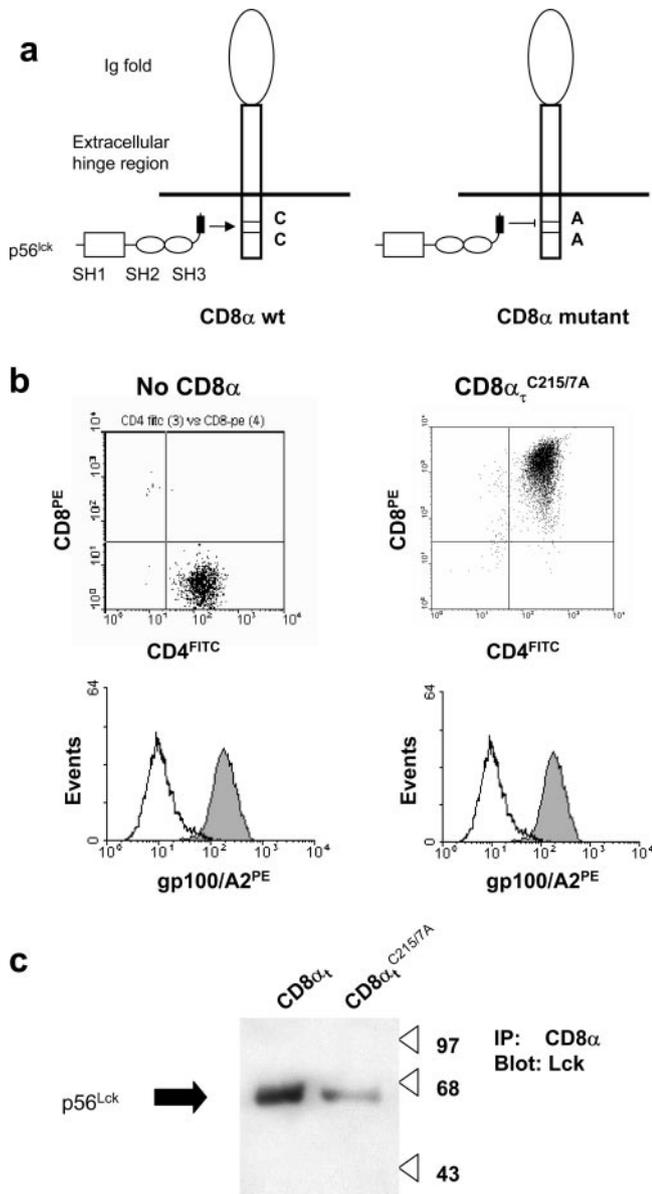
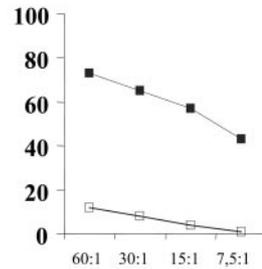


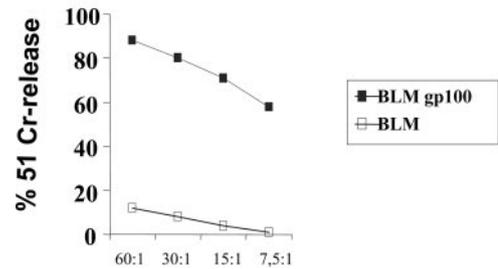
FIGURE 4. Generation and validation of CD8α mutant, termed CD8α_t^{C215/7A}. CD8α_t was mutated at the cysteine motif (CKCP) by changing two cysteines into alanines, yielding CD8α_t^{C215/7A} (a). Mutation of the two cysteines (aa 215 and 217 according to sequence reference number gi 4502688, with amino acid numbering starting at M) prevents binding of p56^{LCK} through its N-terminal aa 10–32 (indicated as ■) (28). Structural domains of both CD8α 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 CD8α_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 CD8α_t (see Fig. 1). c. The decreased ability of CD8α_t^{C215/7A} to recruit LCK is demonstrated by co-immune precipitations. CD4⁺ T cells expressing either CD8α_t or CD8α_t^{C215/7A}, while growing at log phase under normal culture conditions, were lysed and used for CD8α 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).

be relieved by coinjection 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

CD4, gp100/A2 TCR



CD4, CD8α_t, gp100/A2 TCR



CD4, CD8α_t^{C215/7A}, gp100/A2 TCR

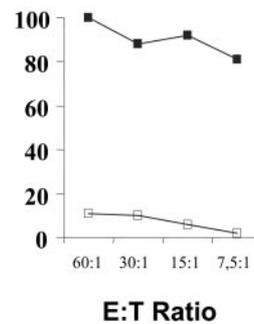
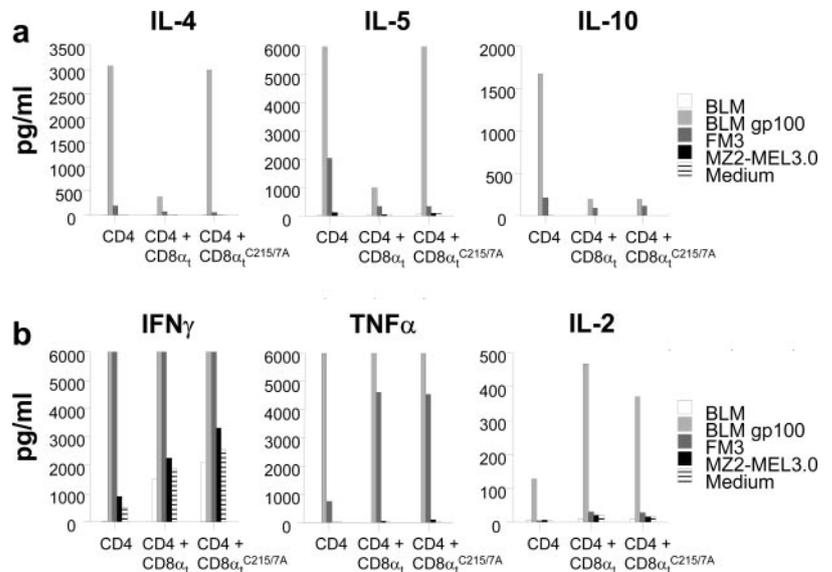


FIGURE 5. CD8α_t^{C215/7A} does not adversely affect tumor-specific T cell cytotoxicity. Three T cell populations expressing gp100/A2 TCR and either no CD8α, CD8α_t, or CD8α_t^{C215/7A} (see Figs. 1 and 4 for the corresponding flow cytometry data) were used as effector T cells in ⁵¹Cr release assays. 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.

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-redirection 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 down-regulates 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. CD8 α -mediated down-regulation of gp100-specific production of IL-4 and IL-5, but not IL-10, depends on the ability of CD8 α 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 α : 107.5/59.2; CD4 + CD8 α ^{C215/7A}: 80.6/48.3. *TNF* α – CD4 + CD8 α ^{C215/7A}: 32.2. *IL*-5 – CD4 + CD8 α ^{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 CD8 α 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). CD8 α 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 CD8 α into TCR-modified CD4⁺ T cells may not be restricted to certain TCRs.

CD8 α containing a mutated LCK and LAT binding domain, termed CD8 α ^{C215/7A}, enabled analysis of the CD8-mediated signaling with respect to down-regulation of IL-4, IL-5, and IL-10 production. The CD8 α 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 CD8 α do, in contrast to CD4, not completely abolish its association with LCK, thereby confirming the initial biochemical characterization of this CD8 α mutant (28). The mutational effect, however, is reported to drastically decrease CD8 α -associated kinase activity (28). Genetic introduction of CD8 α ^{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- γ 1 (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- γ 1, 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 CD8 α -mediated signaling on IL-4, IL-5, and IL-10 production appear not to hold true for endogenous CD8 α (see Fig. 3). We postulate that the apparent differences between exogenous and endogenous CD8 α 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, CD8 β (not present on TCR/CD8 α -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 CD8 α -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 CD8 α -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 α 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 α 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 γ -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

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References

- Dudley, M. E., J. R. Wunderlich, P. F. Robbins, J. C. Yang, P. Hwu, D. J. Schwartztruber, S. L. Topalian, R. Sherry, N. P. Restifo, A. M. Hübicki, et al. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298: 850–854.
- Yee, C., J. A. Thompson, D. Byrd, S. R. Riddell, P. Roche, E. Celis, and P. D. Greenberg. 2002. Adoptive T cell therapy using antigen-specific CD8⁺ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc. Natl. Acad. Sci. USA* 99: 16168–16173.
- Rooney, C. M., C. A. Smith, C. Y. Ng, S. K. Loftin, J. W. Sixbey, Y. Gan, D. K. Srivastava, L. C. Bowman, R. A. Krance, M. K. Brenner, and H. E. Heslop. 1998. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 92: 1549–1555.
- Comoli, P., M. Labirio, S. Basso, F. Baldanti, P. Grossi, M. Furione, M. Viganò, R. Fiocchi, G. Rossi, F. Ginevri, et al. 2002. Infusion of autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. *Blood* 99: 2592–2598.
- Peggs, K. S., S. Verfuert, A. Pizzey, N. Khan, M. Guiver, P. A. Moss, and S. Mackinnon. 2003. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet* 362: 1375–1377.
- Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25⁺CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
- Tada, T., S. Ohzeki, K. Utsumi, H. Takiuchi, M. Muramatsu, X. F. Li, J. Shimizu, H. Fujiwara, and T. Hamaoka. 1991. Transforming growth factor- β -induced inhibition of T cell function: susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. *J. Immunol.* 146: 1077–1082.
- Hunziker, L., P. Klenerman, R. M. Zinkernagel, and S. Ehl. 2002. Exhaustion of cytotoxic T cells during adoptive immunotherapy of virus carrier mice can be prevented by B cells or CD4⁺ T cells. *Eur. J. Immunol.* 32: 374–382.
- Marzo, A. L., B. F. Kinnear, R. A. Lake, J. J. Frelinger, E. J. Collins, B. W. Robinson, and B. Scott. 2000. Tumor-specific CD4⁺ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. *J. Immunol.* 165: 6047–6055.
- Antony, P. A., C. A. Piccirillo, A. Akpınarlı, S. E. Finkelstein, P. J. Speiss, D. R. Surman, D. C. Palmer, C. C. Chan, C. A. Klebanoff, W. W. Overwijk, et al. 2005. CD8⁺ T cell immunity against a tumor/self-antigen is augmented by CD4⁺ T helper cells and hindered by naturally occurring T regulatory cells. *J. Immunol.* 174: 2591–2601.
- Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480–483.
- Surman, D. R., M. E. Dudley, W. W. Overwijk, and N. P. Restifo. 2000. Cutting edge: CD4⁺ T cell control of CD8⁺ T cell reactivity to a model tumor antigen. *J. Immunol.* 164: 562–565.
- Ibe, S., Z. Qin, T. Schuler, S. Preiss, and T. Blankenstein. 2001. Tumor rejection by disturbing tumor stroma cell interactions. *J. Exp. Med.* 194: 1549–1559.
- Corthay, A., D. K. Skovseth, K. U. Lundin, E. Rosjo, H. Omholt, P. O. Hofgaard, G. Haraldsen, and B. Bogen. 2005. Primary antitumor immune response mediated by CD4⁺ T cells. *Immunity* 22: 371–383.
- Willemsen, R., C. Ronteltap, M. Heuveling, R. Debets, and R. Bolhuis. 2005. Redirecting human CD4⁺ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR $\alpha\beta$ gene transfer requires CD8 α . *Gene Ther.* 12: 140–146.
- Chamoto, K., T. Tsuji, H. Funamoto, A. Kosaka, J. Matsuzaki, T. Sato, H. Abe, K. Fujio, K. Yamamoto, T. Kitamura, et al. 2004. Potentiation of tumor eradication by adoptive immunotherapy with T-cell receptor gene-transduced T-helper type 1 cells. *Cancer Res.* 64: 386–390.
- Gao, G. F., J. Tormo, U. C. Gerth, J. R. Weyer, A. J. McMichael, D. I. Stuart, J. I. Bell, E. Y. Jones, and B. K. Jakobsen. 1997. Crystal structure of the complex between human CD8 $\alpha\alpha$ and HLA-A2. *Nature* 387: 630–634.
- Weyer, J. R., B. E. Willcox, G. F. Gao, U. C. Gerth, S. J. Davis, J. I. Bell, P. A. van der Merwe, and B. K. Jakobsen. 1999. T cell receptor and coreceptor CD8 $\alpha\alpha$ bind peptide-MHC independently and with distinct kinetics. *Immunity* 10: 219–225.
- Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by $\alpha\beta$ T cell receptors. *Annu. Rev. Immunol.* 16: 523–544.
- Sewell, A. K., U. C. Gerth, D. A. Price, M. A. Purbhoo, J. M. Boulter, G. F. Gao, J. I. Bell, R. E. Phillips, and B. K. Jakobsen. 1999. Antagonism of cytotoxic T-lymphocyte activation by soluble CD8. *Nat. Med.* 5: 399–404.
- Hutchinson, S. L., L. Wooldridge, S. Tafuro, B. Laugel, M. Glick, J. M. Boulter, B. K. Jakobsen, D. A. Price, and A. K. Sewell. 2003. The CD8 T cell coreceptor exhibits disproportionate biological activity at extremely low binding affinities. *J. Biol. Chem.* 278: 24285–24293.
- Purbhoo, M. A., J. M. Boulter, D. A. Price, A. L. Vuidepot, C. S. Hourigan, P. R. Dunbar, K. Olson, S. J. Dawson, R. E. Phillips, B. K. Jakobsen, et al. 2001. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor ζ chain. *J. Biol. Chem.* 276: 32786–32792.
- Bosselut, R., W. Zhang, J. M. Ashe, J. L. Kopacz, L. E. Samelson, and A. Singer. 1999. Association of the adaptor molecule LAT with CD4 and CD8 coreceptors identifies a new coreceptor function in T cell receptor signal transduction. *J. Exp. Med.* 190: 1517–1526.
- Kuball, J., F. W. Schmitz, R. H. Voss, E. A. Ferreira, R. Engel, P. Guillaume, S. Strand, P. Romero, C. Huber, L. A. Sherman, and M. Theobald. 2005. Cooperation of human tumor-reactive CD4⁺ and CD8⁺ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. *Immunity* 22: 117–129.
- Van de Griend, R. J., B. A. Van Krimpen, S. J. Bol, A. Thompson, and R. L. Bolhuis. 1984. Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. *J. Immunol. Methods* 66: 285–298.
- Schaft, N., R. A. Willemsen, J. de Vries, B. Lankiewicz, B. W. Essers, J. W. Gratama, C. G. Figdor, R. L. Bolhuis, R. Debets, and G. J. Adema. 2003. Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of engineered TCR $\alpha\beta$ genes into primary human T lymphocytes. *J. Immunol.* 170: 2186–2194.
- Willemsen, R. A., M. E. Weijtens, C. Ronteltap, Z. Eshhar, J. W. Gratama, P. Chames, and R. L. Bolhuis. 2000. Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Ther.* 7: 1369–1377.
- Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56^{ck} with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell* 60: 755–765.
- Weijtens, M. E., R. A. Willemsen, E. H. Hart, and R. L. Bolhuis. 1998. A retroviral vector system 'STITCH' in combination with an optimized single chain

- antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes. *Gene Ther.* 5: 1195–1203.
30. Kerry, S. E., J. Buslepp, L. A. Cramer, R. Maile, L. L. Hensley, A. I. Nielsen, P. Kavathas, B. J. Vilen, E. J. Collins, and J. A. Frelinger. 2003. Interplay between TCR affinity and necessity of coreceptor ligation: high-affinity peptide-MHC/TCR interaction overcomes lack of CD8 engagement. *J. Immunol.* 171: 4493–4503.
 31. Vergelli, M., B. Hemmer, P. A. Muraro, L. Tranquill, W. E. Biddison, A. Sarin, H. F. McFarland, and R. Martin. 1997. Human autoreactive CD4⁺ T cell clones use perforin- or Fas/Fas ligand-mediated pathways for target cell lysis. *J. Immunol.* 158: 2756–2761.
 32. Tsuji, T., M. Yasukawa, J. Matsuzaki, T. Ohkuri, K. Chamoto, D. Wakita, T. Azuma, H. Niiya, H. Miyoshi, K. Kuzushima, et al. 2005. Generation of tumor-specific, HLA class I-restricted human Th1 and Tc1 cells by cell engineering with tumor peptide-specific T-cell receptor genes. *Blood* 106: 470–476.
 33. Morris, E. C., A. Tsallios, G. M. Bendle, S. A. Xue, and H. J. Stauss. 2005. A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. *Proc. Natl. Acad. Sci. USA* 102: 7934–7939.
 34. Del Prete, G. 1998. The concept of type-1 and type-2 helper T cells and their cytokines in humans. *Int. Rev. Immunol.* 16: 427–455.
 35. Al Ramadi, B. K., T. Nakamura, D. Leitenberg, and A. L. Bothwell. 1996. Deficient expression of p56^{lck} in Th2 cells leads to partial TCR signaling and a dysregulation in lymphokine mRNA levels. *J. Immunol.* 157: 4751–4761.
 36. Veldhoen, M., A. I. Magee, M. N. Penha-Goncalves, and B. Stockinger. 2005. Transduction of naive CD4 T cells with kinase-deficient Lck-HIV-Tat fusion protein dampens T cell activation and provokes a switch to regulatory function. *Eur. J. Immunol.* 35: 207–216.
 37. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2: 933–944.
 38. Nishimura, T., K. Iwakabe, M. Sekimoto, Y. Ohmi, T. Yahata, M. Nakui, T. Sato, S. Habu, H. Tashiro, M. Sato, and A. Ohta. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J. Exp. Med.* 190: 617–627.
 39. Yu, C. L., R. Jove, and S. J. Burakoff. 1997. Constitutive activation of the Janus kinase-STAT pathway in T lymphoma overexpressing the Lck protein tyrosine kinase. *J. Immunol.* 159: 5206–5210.
 40. Lund, T. C., C. Coleman, E. Horvath, B. M. Sefton, R. Jove, M. M. Medveczky, and P. G. Medveczky. 1999. The Src-family kinase Lck can induce STAT3 phosphorylation and DNA binding activity. *Cell. Signal.* 11: 789–796.
 41. Schwartzberg, P. L., L. D. Finkelstein, and J. A. Readinger. 2005. TEC-family kinases: regulators of T-helper-cell differentiation. *Nat. Rev. Immunol.* 5: 284–295.
 42. Aguado, E., S. Richelme, S. Nunez-Cruz, A. Miazek, A. M. Mura, M. Richelme, X. J. Guo, D. Sainy, H. T. He, B. Malissen, and M. Malissen. 2002. Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. *Science* 296: 2036–2040.
 43. Kovacs, B., M. V. Maus, J. L. Riley, G. S. Derimanov, G. A. Koretzky, C. H. June, and T. H. Finkel. 2002. Human CD8⁺ T cells do not require the polarization of lipid rafts for activation and proliferation. *Proc. Natl. Acad. Sci. USA* 99: 15006–15011.
 44. Arcaro, A., C. Gregoire, T. R. Bakker, L. Baldi, M. Jordan, L. Goffin, N. Boucheron, F. Wurm, P. A. van der Merwe, B. Malissen, and I. F. Luescher. 2001. CD8 β endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56^{lck} complexes. *J. Exp. Med.* 194: 1485–1495.
 45. Huang, Z., J. M. Coleman, Y. Su, M. Mann, J. Ryan, L. D. Shultz, and H. Huang. 2005. SHP-1 regulates STAT6 phosphorylation and IL-4-mediated function in a cell type-specific manner. *Cytokine* 29: 118–124.
 46. Gyobu, H., T. Tsuji, Y. Suzuki, T. Ohkuri, K. Chamoto, M. Kuroki, H. Miyoshi, Y. Kawarada, H. Katoh, T. Takeshima, and T. Nishimura. 2004. Generation and targeting of human tumor-specific Tc1 and Th1 cells transduced with a lentivirus containing a chimeric immunoglobulin T-cell receptor. *Cancer Res.* 64: 1490–1495.
 47. Moeller, M., N. M. Haynes, M. H. Kershaw, J. T. Jackson, M. W. Teng, S. E. Street, L. Cerutti, S. M. Jane, J. A. Trapani, M. J. Smyth, and P. K. Darcy. 2005. Adoptive transfer of gene-engineered CD4⁺ helper T cells induces potent primary and secondary tumor rejection. *Blood* 106: 2995–3003.
 48. Moeller, M., N. M. Haynes, M. H. Kershaw, J. T. Jackson, M. W. Teng, S. E. Street, L. Cerutti, S. M. Jane, J. A. Trapani, M. J. Smyth, and P. K. Darcy. 2005. Adoptive transfer of gene-engineered CD4⁺ helper T cells induces potent primary and secondary tumor rejection. *Blood* 106: 2995–3003.
 49. Debets, R., R. Willemsen, and R. Bolhuis. 2002. Adoptive transfer of T-cell immunity: gene transfer with MHC-restricted receptors. *Trends Immunol.* 23: 435–436.
 50. Couzin, J., and J. Kaizer. 2005. Gene therapy: as Gelsinger case ends, gene therapy suffers another blow. *Science* 307: 1028.