CD8 engineered cytotoxic T cells reprogram melanoma tumor environment

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ABSTRACT
Cytotoxic T lymphocytes (CTL) from CD8\textsuperscript{-}-deficient mice have powerful FasL-mediated cytotoxicity and IFN\textgamma responses, but ablated Ca\textsuperscript{2+} and NFAT signaling, which can be restored by transduction with CD8\beta. Upon infection with lymphocytic choriomeningitis virus (LCMV), these cells yielded GP33-specific CTL (CD8\betaR) that exhibited high FasL/Fas-mediated cytotoxicity, IFN\textgamma CXCL9 and 10 chemokine responses. Transfer of these cells in B16-GP33 tumor bearing mice resulted in (i) massive T cell tumor infiltration, (ii) strong reduction of myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg) and IL-17-expressing T helper cells, (iii) maturation of tumor-associated antigen-presenting cells and (iv) production of endogenous, B16 melanoma-specific CTL that eradicated the tumor long after the transferred CD8\betaR CTL perished. Our study demonstrates that the synergistic combination of strong Fas/FasL mediated cytotoxicity, IFN\textgamma and CXCL9 and 10 responses endows adoptively transferred CTL to reprogram the tumor environment and to thus enable the generation of endogenous, tumoricidal immunity.

Introduction
Melanoma is a highly malignant cancer, characterized by rapid progression, and induction of T cells specific for melanoma antigens like Melan-A/Mart-1, GP-100, tyrosinase and the tyrosinase-related molecules Trp-1 and Trp-2.\textsuperscript{1,2} Cancer progression relies on evasion or suppression of endogenous immunity mediated e.g. by CD4\textsuperscript{+} FoxP3\textsuperscript{+} Treg that secrete IL-10 and transforming growth factor \(\beta\) (TGF\(\beta\)), inhibit inflammatory cytokines, lymphocyte proliferation, upregulate PD-1 on activated T cells, which upon binding to PDL-1 ligands in tumors inhibit CD8\textsuperscript{+} T cells and promote angiogenesis.\textsuperscript{1,3,4} Moreover, MDSC secrete tryptophan-depleting indoleamine 2,3-dioxygenase, ROS and NO radicals, causing T cell dysfunction and apoptosis.

Dendritic cells (DC) play critical roles in immunity and tolerance.\textsuperscript{5,6} In immune-suppressive tumor environments, DC are immature and their tumor antigen presentation induces Treg. In the presence of IFN type I, IFN\textgamma or TNF\alpha, they mature to professional antigen-presenting cells (APC) that express high levels of co-stimulatory CD80, CD86 and CD40 and produce IL-12. IL-12 promotes the formation of Th1 cells, stimulates IFN\gamma and TNF\alpha production and the cytotoxicity of T and NK cells and has been used in cancer gene therapy, typically in combination with lymphoablative pretreatments.\textsuperscript{7,11} IFN\gamma is produced by NK and NKT, Th1 cells and CD8\textsuperscript{+} CTL and upregulates MHC class I and class II molecules, costimulatory CD80 and CD86 molecules as well as the production of the chemokines CXCL9-11.\textsuperscript{12} The cellular composition of a tumor environment is determined in part by the expression of chemokines and their receptors on tumor-infiltrated lymphocytes (TIL) and tumor stroma cells, and depending on the context can support tumor progression or regression.\textsuperscript{12,13}

The aggressively growing B16 mouse melanoma is widely used for preclinical studies.\textsuperscript{13,14} Upon injection of B16 cells into C57BL/6 (B6) mice, endogenous T cells emerge that are specific mainly for Trp1, Trp2 and GP100 and fail to control tumor growth. In some studies, surrogate tumor antigens, like OVA or LCMV glycoprotein GP33 were used in adoptive CTL transfer experiments, resulting in transient tumor control, but rarely complete eradication.\textsuperscript{3,4,15} By contrast, transfer of CD8\textsuperscript{+} T cells transduced with high avidity T cell receptors (TCR) or chimeric antigen receptors (CAR) has been shown to mediate complete tumor eradication.\textsuperscript{3,7,9,16,17} Importantly, these therapies to be efficient require harsh pretreatments, like whole body irradiation and/or aggressive chemotherapy. Because for some patients such pretreatments are not applicable, we searched for a strategy to reject established B16 tumors by enabling the induction of endogenous, tumoricidal T cell immunity. We previously observed that CD8\betaKO mice efficiently clear acute LCMV infections, even though their CTL have defective Ca\textsuperscript{2+} mobilization and perforin/granzyme-mediated killing.\textsuperscript{18,19} Using mice expressing transgene TCR\beta chain of the LCMV D\textsuperscript{b}/GP33-specific P14 TCR, we showed that LCMV induced, D\textsuperscript{b}/GP33-specific CTL from CD8\betaKO mice have normal IFN\gamma responses, but greatly increased Fas/FasL-mediated cytotoxicity, both triggered via a Ca\textsuperscript{2+} independent, PI3K and PKC\delta dependent signaling pathway.\textsuperscript{19,21} By contrast, perforin/granzyme-dependent killing and IFN\gamma production by “conventional” CTL relied on CD3 phosphorylation by CD8-associated p56\textsuperscript{ck}, activation of PLC\gamma, strong Ca\textsuperscript{2+} mobilization, NFAT activation and nuclear translocation.\textsuperscript{19,22} Importantly, Fas/FasL-mediated cytotoxicity plays critical and non-redundant roles in tumor rejection.\textsuperscript{7,8,23-25}
We report that introduction of CD8β in CD8α+ T cells from CD8βKO mice and LCMV infection allows the generation of GP33-specific CTL (CD8βR) with greatly enhanced IFNγ responses and Fas/FasL-mediated cytotoxicity. Adoptive transfer of CD8βR CTL resulted in complete and lasting eradication of B16-GP33 tumors in most mice and formation of a protective memory response. These CTL reprogrammed the microenvironment enabling the generation of endogenous B16 tumor-specific CTL, capable of eradicating the tumor long after the transferred CTL perished.

Results

CD8βR CTL exhibit restored Ca2+ mobilization and degranulation

We examined whether transfection of CD8α+ splenocytes from CD8βKO P14 TCRβ transgenic mice with CD8β restored their defective intracellular Ca2+ mobilization and degranulation. The transduced CD45.2+ T cells were injected into CD45.1+ recipients, infected with LCMV and after 8 d flow cytometry analysis of their splenocytes indicated high frequencies of CD45.2+, Dβ/GP33 tetramer positive CTL (Figs. 1A–C). The CTL derived from P14 TCRβ transgenic are referred to as WT, those from P14 TCRβ CD8β/KO mice as KO and those transduced with CD8β as CD8βR. All CTL exhibited comparable CD44 and CD127 expression, but CD8βR CTL increased CD5, CD25 and CD69 expression, suggesting that they were more activated (Fig. S1). Furthermore, CD8βR CTL efficiently fluxed Ca2+, even surpassing WT CTL at low GP33 peptide concentrations, whereas KO CTL were refractory (Fig. 1D). Similarly, CD8βR CTL displayed higher CD107a expression than WT and especially KO CTL (Figs. 1E, F). 45 d after LCMV infection all CTL contracted by approximately 95%, arguing that CD8βR CTL are not auto-reactive (Fig. 1C).

CD8βR CTL exhibit increased IFNγ response

To characterize the CTL under study, we assessed their cytokine responses. The TNFα response was similar for KO, CD8β/R and WT CTL, whereas the IFNγ response of CD8β/R CTL was 30–40% higher compared to KO CTL, both in terms of percent of IFNγ+ cells and MFI and 20% higher compared to WT CTL in terms of IFNγ+ cells (Figs. 2A–F). The GP33 peptide concentration giving half-maximal IFNγ response (IC50) was over 10-fold lower for CD8βR compared to WT CTL and 5-fold lower compared to KO CTL. In the presence of the PI3K inhibitor Wortmannin, the IFNγ response of KO CTL was ablated, the one of WT CTL modestly and the one of CD8βR CTL substantially inhibited. This was consistent with the observation that WT and KO CTL use two different IFNγ signaling pathways and indicated that CD8βR CTL can use both.

CD8β/R CTL are highly cytotoxic

We next examined the CTL’s expression of granzyme and perforin. CD8βR and WT CTL exhibited comparable perforin expressions, both higher compared to those of KO CTL (Figs. 3A, C). However, the granzyme B expression was significantly higher on CD8βR CTL than on WT or KO CTL (Figs. 3B, D). Conversely, the surface expression of CD95L (eCD95L) of KO CTL was dramatically higher compared to WT CTL both in terms of MFI and proportion of positive cells (Figs. 3E, G). For CD8β/R CTL, the proportion of eCD95L positive cells was similar compared to KO CTL, but higher in terms of MFI. The intracellular CD95L expression (iCD95L) of CD8β/R CTL was higher compared to WT CTL and KO CTL (Figs. 3F, H). These results indicated that CD8β transduction restored the perforin and granzyme B expression of KO CTL, but did not diminish their high Fasl expression.

We next compared the cytotoxicity of KO, CD8β/R and WT CTL using GP33 peptide pulsed 51Cr labeled Dβ/P815 cells as targets. Maximal lysis was observed for CD8β/R CTL (87.5%), followed by WT CTL (55%) and KO CTL (27.7%) (Fig. 3I). The corresponding IC50 values were similar (range 0.96–1 × 10−9 M). Blocking anti-FasL antibody inhibited lysis to different degrees; for KO CTL, the inhibition was complete, for CD8β/R about 25% and for WT CTL 14–16%. The corresponding IC50 value varied in the range of 0.2–1 × 10−3 M. Conversely, concanamycin A (CMA), a blocker of perforin/granzyme-mediated killing, inhibited the CD8β/R CTL-mediated lysis by over 50%, had no effect on KO CTL, but abolished WT CTL-mediated killing (Fig. 3J). The corresponding IC50 values varied little (range 0.3–0.7 × 10−3 M). GP33 transduced B16 melanoma (B16-GP33) cells were then used as targets at different E/T ratios. Maximal lysis was observed for CD8β/R (about 63%), followed by KO (43%) and WT (40%) (Fig. 3K). Because IFNγ upregulated MHC class I and II and CD80 on B16-GP33 cells (Fig. S2A), this experiment was repeated with IFNγ-pretreated B16-GP33 cells. Maximal lysis, nearly 100%, was observed for CD8β/R CTL, 75% and 60% for WT and KO CTL respectively (Fig. 3K). The same experiment performed with B16 cells indicated that the non-specific lysis increased with the E/T ratio, was highest for CD8β/R CTL on IFNγ-pretreated cells (17% lysis) and smallest for KO CTL (6%) (Fig. 3L).

CD8β/R, but not WT or KO CTL eradicate established B16-GP33 tumors

To assess the ability of CD8β/R, KO and WT CTL to control tumors, they were adoptively transferred into B16-GP33 tumor-bearing mice and tumor growth monitored over time (Fig. 4A). Transfer of 105 CD8β/R CTL resulted in complete tumor eradication in 7 of 13 mice (Figs. 4B–D). During the first 18 d after transfer, there was scant tumor growth, followed 30–40 d later by complete tumor eradication. Escapes were observed after 20–40 d and a few ones as late as 50 d post transfer. Importantly, none of the B16-GP33 tumor-bearing mice inoculated with WT or KO CTL survived. Even more transient tumor control was observed in B16 tumor-bearing hosts (Fig. 4B). Using a different protocol, we tested the ability of CD8β/R CTL to prevent lung tumor formation. When mice were injected i.v. with 106 B16-GP33 cells and three days later with 105 CD8β/R CTL, their lungs exhibited substantially less lung
tumors than those injected with KO or WT CTL, as judged visually, by tumor surface and lung weight (Fig. S3). Finally, we tested whether the long-term surviving mice (Figs. 4B, D) had a protective memory, they were injected i.v. with B16-GP33 cells. Three weeks later, their lungs exhibited barely detectable B16-GP33 tumor, whereas those of control mice were full of tumors (Figs. 4E–G).

**CD8βR CTL foster endogenous tumor-specific CD8+ T cell immunity**

To gain mechanistic understanding, B16-GP33 tumors were excised after adoptive transfer of CD8βR or WT CTL and examined by microscopy and flow cytometry. After 16 h, comparable numbers of transferred CD8βR or WT CTL
were found in the tumors (Fig. 5J). Sections of tumors from mice inoculated with CD8βR CTL exhibited rare, large clusters of CD45.1+ cells, about one-third of which were CD8+ cells intermingled with CD45.1-CD8+ cells and numerous CD8- cells (Figs. 5A–C, J). The CD8+ T cells comprised endogenous (CD45.1+) B16 tumor antigen-specific T cells; mostly Trp-2 specific ones and exogenous, transferred CD8βR CTL (CD45.1+)(Figs. 5J, K). The endogenous CD8- cells contained among others CD4+ T cells and CD11c+ APC (Figs. 6A–D, G, I–M). Sections of tumors from mice injected with WT CTL exhibited smaller and less dense clusters of CD45.1+ cells and these contained less CD8+ cells, as confirmed by flow cytometry and indicating that WT CTL promoted less CD8+ T cell tumor infiltration than CD8βR CTL (Figs. 5D, E, J, K). After 15 d of CD8βR CTL transfer, tumor sections exhibited 30–40% of CD45.1+CD8+ T cells, which as indicated by flow cytometric analysis were in part B16 melanoma antigen-specific CTL (Figs. 5F, G, J, K). By contrast, sections of tumors from mice injected 15 d previously with WT CTL displayed only 15–20% of endogenous CD8+ T cells among the CD45.1+ cells and lower frequencies of melanoma antigen-specific CTL were observed by flow cytometry (Figs. 5H–K). In these tumors, substantially higher numbers of CD45.1-, CD8+ cells, i.e. transferred CD45.2+ CTL, were detected compared to tumors from mice injected with CD8βR CTL.

Figure 2. CD8βR CTL exhibit increased IFNγ responses. (A–F) Eight days after LCMV infection splenocytes containing KO (orange lines), CD8βR (green lines) or WT CTL (blue lines) were incubated with 1 μM GP33 peptide for 4 h and intracellular TNFα (A) or IFNγ (D) expressions assessed by flow cytometry by gating on CD45.2+, D3/GP33 tetramer+ cells. The shaded histogram is the isotype control. Alternatively, graded concentrations of GP33 peptide were used and the percentages (B, E) or mean fluorescence intensity (MFI) (C, F) of TNFα (B, C) or IFNγ (E, F) T cells enumerated. Hundred percent refers to CD45.2+, D3/GP33 tetramer+ cells. Mean values and SEM were calculated from 2–3 experiments. (G) Intracellular IFNγ expression of CD45.2+, D3/GP33 CTL was assessed after 4 h incubation with graded GP33 peptide concentrations in the absence (solid lines) or presence (dashed lines) of Wortmannin (100 nM). Mean values and SEM were calculated from two experiments. (H) The corresponding IC50 values i.e., GP33 peptide concentrations (pM) giving half maximal response and the maximal values (Max) are represented.
Melanoma antigen-specific CD45.1$^+$, CD8$^+$ CTL were also observed in B16-GP33 tumor draining lymph nodes (Fig. S4A). Shortly after transfer, 7.5–10% of these CTL were Trp-2-specific and only after 15 d substantial frequencies of other specificities were observed (17% GP33, 10% GP100 and 4% Trp-1-specific CTL). In lymph nodes from mice inoculated
with WT CTL, the frequencies of these cells were only 0.8–2.5%. Analysis of splenocytes from the same mice showed a similar scenario, but at considerably lower frequencies (Fig. S4D).

We next characterized the CD45.2+ CD8βR and WT CTL recovered from B16-GP33 tumors 4 d post transfer. The PD-1 expression of tumor-infiltrated CD8βR cells was markedly lower compared to WT CTL, whereas the CD44 expressions were essentially the same (Fig. 5L, S5). The PD-1 expression of CD45.1+, CD8+ TIL 4 d and 15 d after transfer of CD8βR CTL was substantially lower compared to those from mice injected with WT CTL (Figs. 5M, N; S5A). The CD44 expression of CD45.1+, CD8+ TIL 4 d post transfer was biphasic in both groups, with the CD44<sup>high</sup> fraction being higher in the CD8βR group; after 15 d nearly all cells in the CD8βR group were CD44<sup>high</sup>, whereas most cells in the WT group were CD44<sup>low</sup> (Figs. 5N, S5B).

To identify the cytokines and chemokines produced by CTL and tumor cells, we incubated CD8βR and WT CTL with B16-GP33 cells in vitro and performed intracellular cytokine staining. Remarkably, nearly half of the CD8βR CTL were CXCL9<sup>+</sup>, i.e. 3–4-fold more compared to WT CTL (Fig. 5O). A smaller difference and lower expression levels were observed for CCL2, whereas the inverse was true for CCL3. Analogous but smaller differences were observed for CTL without incubation with B16-GP33 cells. B16-GP33 cells upon incubation with the CTL

**Figure 4.** CD8βR CTL eradicate established B16-GP33 tumors and protect mice against rechallenge. (A) CD45.1<sup>+</sup> mice were injected s.c. with 10<sup>5</sup> B16 or GP33<sup>+</sup> B16 melanoma cells; after palpable tumors were formed, 10<sup>6</sup> CD45.2<sup>+</sup> KO (orange), CD8βR (green) or WT (blue) CTL were injected i.v. (day 0) and tumor volumes measured over time. (B) Average volumes of GP33<sup>+</sup> B16 (solid lines) or B16 (dashed lines) tumors observed on different days after CTL transfer. Mean values and SEM were calculated from 13 mice. (C) GP33<sup>+</sup> B16 tumor growth curves of individual mice for the experiment shown in B. (D) Kaplan Meier plots for 13 mice per group. (E–G) The mice exhibiting complete tumor eradication were injected i.v. 10<sup>6</sup> GP33<sup>+</sup> B16 cells 200 d after the transfer of CTL and 21 d later their lungs were isolated, photographed (E) and their weight (F) and tumor surface measured (G). Mean values and SD were calculated from two experiments. The <i>p</i> value indicates **<i>p</i> < 0.01. Control refers to untreated mice.
Figure 5. Analysis of tumor-infiltrated T cells. (A–I) B16-GP33 tumor-bearing CD45.1\(^{+}\) mice were inoculated with \(10^5\) CD45.1\(^{+}\) CD8\(/\)R (left) or WT CTL (right); after 16 h (A–E) or 15 d (F–I) tumor sections were analyzed by microscopy upon staining with antibodies specific for CD45.1 (green), CD8\(/\) (red) and fibronectin (blue). (A) and (B) show two different sections; zooms the enlargements of the white boxes and white bars the scales. (J) GP33\(^{+}\) B16 tumors were excised at different times after CTL transfer (x-axis) and their single cell suspensions analyzed by flow cytometry for their contents of transferred CD45.2\(^{+}\), D\(^{b}\)/GP33\(^{+}\) CD8\(/\)R (dark green circles), WT CTL (dark blue squares) or endogenous, CD45.1\(^{+}\), CD8\(^{+}\) T cells after transfer of CD8\(/\)R (light green squares) or WT CTL (light blue circles). The y-axis represents the number of CD8\(^{+}\) T cells per mg tumor. (K) The endogenous CD45.1\(^{+}\), CD8\(^{+}\) T cells were stained with tetramers specific for GP100 (squares; dashed pointed lines), Trp1 (triangles, solid lines), Trp2 (diamonds, double pointed dashed lines) and GP33 (circles, dashed lines) and the tetramer positive cells enumerated by flow cytometry. The mean values and SD were calculated from two experiments. (L, M) Endogenous CD45.1\(^{+}\), CD8\(^{+}\) T cells in tumors from mice 4 d (L) or 15 d (M) previously inoculated with CD8\(/\)R (light green) or WT CTL (light blue) were analyzed likewise. (O, P) Freshly isolated CD8\(/\)R (green circles) or WT CTL (blue squares) were incubated with GP33\(^{+}\) B16 cells (1:1) for 6 h; the cells were intracellularly stained with antibodies specific for the indicated cytokines and analyzed by flow cytometry, gating on CTL (O) or GP33\(^{+}\) B16 cells (P). Each symbol represents a mouse, the inserted lines mean values and SEM and the bars the values measured after 6 h of incubation in medium only.
Figure 6. Analysis of tumor infiltrated CD4+ T cells. (A–D) GP33+ B16 tumors from mice inoculated 16 h previously with 10^5 CD8bR (A, B) or WT CTL (C, D) were excised and analyzed by microscopy after staining of sections with antibodies specific for FoxP3 (green), CD4 (blue) and I-Ab (red). (B) and (D) show the enlargements of the boxed areas in (A) and (C). (E, F) The percentages of Foxp3-CD4+ Treg (green), FoxP3+CD4+ cells (dark blue) T cells, Treg-APC conjugates (light blue) and Foxp3-CD4+APC (pink). Hundred percent refers to the number of CD4+ T cells. (G) Numbers of CD4+ T cells found per mg of B16-GP33 tumor at different days after adoptive transfer of CD8bR (green) or WT (blue) CTL. Mean values and SD were calculated from two experiments. (H) Tumor-infiltrated CD4+ T cells isolated from tumors of mice inoculated at the indicated times previously with CD8bR (green) or WT (blue) CTL were analyzed by flow cytometry upon intracellular staining with antibodies specific for RORγ (Th17), GATA-3 (Th2), FoxP3 (Treg) or T-bet (Th1). The inserted numbers indicate the percentages of cells found in the indicated gates. The x-axes indicate fluorescence intensities and the y-axes counts with maximal being 100%. One of two experiments is shown. (I–L) B16-GP33 tumors from mice inoculated 16 h previously with CD8bR (I, K) or WT (L, M) CTL were analyzed by microscopy upon staining of sections with antibodies specific for CD4 (green), CD11c (red) or fibronectin (blue). (K) and (M) show the enlargements of the white boxes in (I) and (L). (N, O) Pie charts representing the percentages of CD4+ T cells conjugated (yellow) or not (green) with CD11c+ APC in (I) and (L), respectively. Hundred percent refers to total CD4+ T cells. (P) The numbers of CD11c+ APC per mg of tumor from mice inoculated 16 h, 2 d and 4 d previously with CD8bR (green circles) or WT CTL (blue squares), was enumerated by flow cytometry. Mean values and SEM were calculated from two experiments. (Q) Tumor-associated CD11c+ APC were isolated from tumors of mice 16 h previously inoculated with CD8bR (green bars) or WT CTL (blue bars) and analyzed by flow cytometry for expression of CD80, CD40 and I-Ab. Mean values and SEM were calculated from two experiments. (R) The fold changes in the numbers of CD11bC, Gr1C MDSC enumerated by flow cytometry of cell suspensions of tumors (solid lines) or spleens (dashed lines) from mice inoculated at different times previously with CD8bR (green lines) or WT CTL (blue lines). Mean values and SEM were calculated from two experiments. The p values indicate 0 p < .1 and 00 p < .01.
exhibited similar CCL2, CCL3 and CXCL9 expression profiles, but the differences between CD8/R and WT CTL cells were smaller (Fig. 5P). Consistent with this, supernatants of CD8/R but not WT CTL incubations with B16-GP33 cells contained high concentrations of CXCL9 and less CXCL10 (Fig. S6A). Importantly, high proportions of B16-GP33 cells expressed TGFβ and IL-10 upon incubation with WT but not with CD8/R CTL (Fig. 5P). Finally, we assessed chemokine contents of sera of B16-GP33 tumor-bearing mice 15 d after incubation with CD8/R and WT CTL. The concentrations of inflammatory CCL2, 3 and 5 and the IFNγ-induced chemokines CXCL9 and 10 were 2-fold (for CXCL10) to 4000-fold (for CCL5) higher in sera of mice injected with CD8/R CTL (Fig. S6B).

We next assessed the expression of immunologically relevant molecules on B16-GP33 cells isolated from tumors of mice inoculated 15 d previously with CTL. Tumor cells from mice inoculated with CD8/R CTL exhibited substantially higher expressions of I-Aβ and Kσ compared to those injected with WT or KO CTL (Fig. S2B). Fas expression was strongly upregulated in the CD8/R group (74.7%), less in the WT and KO groups (66.8 and 56%), whereas the FasL expression was low in all groups. The upregulation of some but not other of these molecules, especially in the CD8/R group, were reminiscent to those observed on B16 cells upon incubation with IFNγ (Fig. S2A).

Because CTL are sensitive to Fas/FasL-mediated apoptosis,26 we examined the death of ex vivo CD8/R and WT CTL upon incubation with γ-irradiated B16-GP33 cells. At an E/T ratio of 100, about 30% of the CD8/R CTL were apoptotic after 12 h of incubation, whereas for the WT CTL it was only about 12% (Fig. S6C). Half-maximal apoptosis was observed at an E/T ratio of about 2.7 and 14 for CD8/R and WT CTL, respectively. The Fas and FasL expressions of CD8/R CTL isolated from tumors were several folds higher than those of WT CTL (Figs. S6D, E). These findings explain why CD8/R CTL rapidly disappeared in vivo (Fig. 5).}

**CD8/R CTL reprogram the tumor microenvironment**

We next examined tumor-associated CD4+ T cells after transfer of CD8/R or WT CTL into B16-GP33 tumor-bearing hosts. Tumors from mice inoculated with CD8/R CTL 16 h previously contained about three times more CD4+ T cells than those from mice injected with WT CTL (Figs. 6A–D, G). Some CD4+ T cells co-localized with I-Aβ+, CD11c+ APC, i.e. DC and macrophages (Figs. 6A, B, I, K). After 16 h of transfer, approximately 40% of the CD4+ T cells were FoxP3+Treg of which 21.6% were conjugated with I-Aβ+ APC (Figs. 6E, H). The other CD4+ T cells comprised Th1, 10-fold less Th2 and even fewer Th17. Approximately 41% of these other CD4+ T cells were conjugated with I-Aβ+ APC and 20.5% were free. Conversely, tumors of mice injected with WT CTL contained fewer CD4+ T cells, 58.4% of which were FoxP3+ and of these 33.5% conjugated with APC (Figs. 6C, D, F, H). Of the other CD4+ T cells, 31.1% were conjugated with I-Aβ+ APC. Tumor sections stained for CD4 and CD11c, a marker of DC and macrophages, showed both cell types interspersed in tumors from mice inoculated with CD8/R and WT CTL, with a higher fraction of conjugates in the latter group (Figs. 6I–N). At later time points, there was an increase of CD4+ TIL, first more in tumors from mice injected with WT and later in those in injected with CD8/R CTL (Fig. 6G). Importantly, tumors of mice inoculated with CD8/R CTL exhibited a striking time-dependent decrease of Treg and Th17 and to a lesser extent of Th2 cells, whereas the inverse was true for tumors of mice injected with WT CTL (Fig. 6H).

We analyzed likewise CD4+ T cells from tumor draining lymph nodes and found that those from mice inoculated with CD8/R CTL similar proportions of CD4 T cell subtypes as in the in tumors, except that FoxP3+ Treg were more frequent (Fig. S4B). In the lymph nodes of mice injected with WT CTL, the proportions of Th2 and Th17 cells were initially higher compared to TIL (Fig. S4C). Analysis of the spleens showed similar proportions of CD4 T cell subtypes; expect that the fraction of Th2 cells was higher in the CD8/R group (Figs. S4E, F).

Tumors from mice inoculated with CD8/R CTL contained 3–5-fold more CD11c+ APC than those from mice injected with WT CTL (Fig. 6P). Already after 16 h, these APC expressed higher levels of CD80 (B7.1), CD40 and especially I-Aβ, than APC of the other group, i.e., they were more mature (Fig. 6Q). Importantly, upon transfer of CD8/R CTL, the MDSC decreased over 3-fold, but increased by 20-fold upon transfer of WT CTL (Fig. 6R). The inverse trends, albeit weaker, were observed in the spleens, arguing that the changes of tumor-associated MDSC were accounted for in part by migration.

Our findings can be summarized as illustrated in Figure S7; i.e. CD8/R CTL upon encounter with B16-GP33 cells became highly activated resulting in: (i) massive killing of tumor and stroma cells by powerful perforin/granzyme and Fas/FasL-mediated cytotoxicity; (ii) release of extraordinary quantities of IFNγ and angiostatic CXCL9; (iii) rapid and strong reduction of tumor-associated Treg and MDSC; (iv) maturation of APC and (v) tumor infiltration and expansion of B16 tumor-specific CD8+ CTL that eradicate the tumor long after the CD8/R CTL succumbed to their own high cytotoxicity (Figs. S7A, B). Conversely, transfer of WT CTL increased the frequencies of tumor-associated Treg, Th17 and MDSC, thus preventing maturation of APC, infiltration and expansion of endogenous tumor-specific CTL, altogether resulting in uncontrolled tumor growth (Figs. S7C, D).

To validate this concept, we inoculated B16-GP33 tumor-bearing mice with CD8/R CTL that were previously injected with antibodies neutralizing IFNγ or blocking CXCR3, the receptor for the IFNγ induced chemokines CXCL9-11. In both cases, the tumor eradication by CD8/R CTL was abolished (Figs. S8A–C). Flow cytometric analysis of tumor infiltrated cells two days post transfer of CD8/R CTL indicated that neutralization of IFNγ reduced the number of endogenous CD8+ T cells by about 3-fold, the number of CD11c+ APC by 5–6-fold, but increased the counts of CD11b+, Gr1+ MDSC by nearly 10-fold (Fig. S8D). In the CD4 compartment, neutralization of IFNγ increased the number of Treg by 10-fold and decreased the one of Th1 cells by nearly as much, i.e., it reversed the changes induced by transfer of CD8/R CTL (Figs. 6H, S8E). Neutralization of IFNγ decreased the numbers of Th17 and Th2 cells, but the changes were modest. Blocking of CXCR3 had similar effects as neutralization of IFNγ,
suggesting that in this system the IFNγ-induced CXC9-11 chemokines mediated directly or indirectly tumor infiltration and/or proliferation of lymphoid and myeloid cells (Figs. S8D, F).27 Two days after transfer of CD8βR CTL, tumor-resident CD11c+ APC expressed high levels of I-Ak and intracellular IL-12 and MHC II, both of which are required for priming of T cells.5,10 CD11c+ APC exhibit a more mature phenotype two days as compared to 16 h post transfer of CD8βR CTL (Fig. 6O, P, S8D, F).

Discussion

Upon LCMV infection, CD8βKO mice generated GP33-specific CTL with ablated Ca2+ mobilization and perforin/granzyme-dependent cytotoxicity that cleared the infections by relying on powerful FasL/Fas-mediated cytotoxicity (Figs. 1D–F).18,19 The cytotoxicity and IFNγ response of these CTL were signaled by a Ca2+ independent, PI3K, PKCθ-dependent pathway, whereas perforin/granzyme-mediated killing, TNFα and IFNγ responses of "conventional" CTL were triggered mainly by a Ca2+ and NFAT-dependent signaling pathway.19-22 The former pathway may be dominant in CD8α+ T cells from CD8βKO mice due to co-signaling via FasL compensating defective CD8αβ-Ick signaling during T cell development.28 This would explain the greatly increased FasL expression and Fas/FasL-mediated killing of KO and CD8βR CTL (Figs. 3E–J).19

CD8β transduction of CD8α+ T cells from CD8βKO mice and LCMV infection yielded CD8βR CTL with fully restored intracellular Ca2+ mobilization and degranulation (Figs. 1D–F) and modestly increased levels of the activation markers CD5 and CD25 (Fig. S1A). Because after the acute phase of LCMV infection CD8βR CTL contracted like WT and KO CTL, they apparently were not auto-reactive (Fig. 1C). Importantly, CD8βR CTL exhibited substantially higher IFNγ responses compared to WT and KO CTL, because in these cells IFNγ was induced by the conventional Ca2+ NFAT-dependent and the alternative Ca2+ independent, PI3K-dependent signaling pathways (Figs. 2D–H).19 Such high IFNγ production in the tumor microenvironment in our and other studies played crucial roles for its reprogramming and tumor eradication. First, it strongly upregulated MHC class I and class II molecules and CD80 on B16-GP33 cells, thus increasing their immunogenicity and recognition by CTL (Figs. 3K, S2A). IFNγ also promotes tumor antigen cross-presentation by stroma cells, rendering these susceptible to T cell recognition and providing new opportunities for T cell priming.29,30 Second, IFNγ induced high expression of CXCL9 and 10 (Figs. 5O, S6A), chemokines that promoted tumor infiltration of endogenous T cells (Figs. 5A–J, 6A–G).12,27,31,32 These cells were CXCR3+–activated T cells including B16 antigen-specific CD8+ T cells. The importance of this is illustrated by tumor escape mechanism relying on CXCL9 downmodulation.33 Third, IFNγ polarized CD4+ T cells to Th1, explaining why tumors of mice injected with CD8βR CTL have increased Th1 frequencies (Fig. 6H). Because activated Th1 cells produce IFNγ, this contributes to augmenting local CXCL9 (and CXCL10) expression.4,27 Fourth, IFNγ namely via induction of CXCL9 and CXCL10 chemokines is strongly angiostatic and damages the tumor vasculature.8,29,30,34,35 Minimal perivascular T cell infiltrates can initiate vascular destruction inside the tumor, leading to central tumor necrosis.30

The other hallmark of CD8βR CTL was their high Fas/FasL-mediated cytotoxicity that together with restored perforin/granzyme-mediated killing afforded increased lysis of target and tumor cells compared to KO and WT CTL (Figs. 3I–L). Although perforin/granzyme-mediated cytotoxicity allows killing of large numbers of tumor cells, it is susceptible to MDSC and Treg-mediated inhibition, tumor cell resistance to perforin and scant tumor antigen presentation.1,3,36 Conversely, avid Fas/FasL-mediated cytotoxicity relying on high Fas and of FasL expression can eliminate tumor stroma cells including MDSC and Treg and thus strongly contributes to reprogramming of the tumor environment and the course of tumor-specific immunity (Fig. 6).7,8,23-25,37 Fas/FasL-mediated cytotoxicity also accounted for the disappearance of CD8βR CTL a few days after adoptive transfer by fratricide or suicide (Figs. 5J, S6C–E).26 The relatively rapid loss of adoptively transferred tumor-specific CTL limits the risk of potentially deleterious off target effects.

Because CD8βR CTL disappeared long before tumor eradication (Figs. 4A–D, 5J), it was possible to elucidate key components of the tumor environment reprogramming they induced. Already 16 h after transfer of CD8βR, but not WT CTL, we observed: first, massive infiltration of CD45+ CD8+ and CD8– T cells including antigen-specific endogenous CD8+ and CD4+ T cells (Figs. 5A–E, J, K, 6A–G). Their tumor infiltration was promoted by chemotaxis by CXCL9 and CXCL10, abundantly produced by CD8βR CTL encountering B16-GP33 tumor cells, but to a lesser extent also by the melanoma cells (Figs. 5O, P, S6A).12,27,29,33,34 The expression of these chemokines was induced by high local IFNγ production by CD8βR CTL (Fig. 2D–H) favored by reduced Treg frequency (Fig. 6H)26 and by CD8+ TII expressing low PD-1 (Figs. 5L–N, S5A) (31). Consistent with this are the observations that neutralization of IFNγ and blockade of CXCR3 substantially reduced the numbers of tumor-associated endogenous CD8+ T cells and CD11c+ APC (Fig. S8D). Moreover, IFNγ, namely via induction of CXCL9/10 chemokines, has been shown to be strongly angiostatic, and to thus play another important role in tumor eradication.12,27,29,30,34,35 Second, a nearly 9-fold decrease of tumor-associated MDSC was observed, whereas transfer of WT CTL resulted in a 20-fold increase and concomitant inverse, smaller changes in the spleen (Fig. 6R). It has been shown that transfer of tumor-specific conventional CTL fosters tumor immunosuppression and escape via recruitment of MDSC from the periphery into the tumor.1,12,13 The reduction of tumor-associated MDSC observed upon adoptive transfer of CD8βR CTL was accounted for in part by their strong Fas/FasL-mediated cytotoxicity, which has been shown to decimate MDSC (Figs. 3E–J, 6Q)7,39 Because IFNγ increases Fas/FasL-mediated killing,7 this also explains why neutralization of IFNγ increased the number of tumor-associated MDSC (Fig. S8D). In addition, in tumors of mice treated with CD8βR CTL MDSC may differentiated in professional APC.9,33,38,40 Third, a
decrease of tumor-associated Foxp3+ Treg and Treg-APC conjugates, explained by the reduced number of tumor-associated MDSC, which induce Treg and by decimation by FasL-mediated killing by CD8βR CL (Figs. 5P, 6E). Reduced frequencies of Treg in turn increased the IFNγ and CXC9/10 chemokine production and tumor infiltration by CXCR3+ activated T cells (Figs. S8D, E) and decreased expression of immune-suppressive IL-10 and TGFβ. Fourth, we observed a strong decrease of Th17 cell frequency in tumors from CD8βR CTL treated mice and massive increase in tumors from animals injected with WT CTL (Fig. 6H). IL-17 produced by CD4+ Th17 cells has been shown to support tumor growth by upregulating pro-survival and pro-angiogenic genes as well as tumor-associated MDSC and Treg.

These changes became more pronounced at later times after transfer of CD8βR CTL and abolished immune suppression in the tumor environment. For example, CD8+ T cells from tumors of mice injected with CD8βR CTL were PD-1low, whereas those from tumors of mice inoculated with WT CTL were PD-1high (Figs. 5L–N, S5A). Because PD-1 is upregulated by Treg and MDSC and conveys T cell dysfunction, this indicated that transfer of CD8βR, but not WT CTL created a tumor environment permissive to T cell immunity. Importantly, tumors of mice inoculated with CD8βR CTL exhibited three—four times higher numbers of CD11c+ APC and these were mature, whereas those of tumors from mice injected with WT CTL were not (Figs. 6P, Q S8D, F). This was explained in part by massive tumor cell killing by CD8βR CTL, producing vast numbers of dying cells that promote DC maturation by cell-death-associated-molecular-pattern-mediated toll-like receptor triggering of IFN type I and IL-12 production. In addition, maturation of DC was promoted by the high IFNγ production in tumors of mice treated with CD8βR CTL, initially primarily by these cells and later by Th1 cells and endogenous CTL (Figs. 2D–H, S2, 6I). These factors may also induce differentiation of MDSC into CD11c+ myeloid DC and/ or macrophages, explaining the increased frequencies of the mature APC in tumors. Consistent with this is the observation that neutralization of IFNγ reduced not only the number of CD11c+ APC in tumors form mice treated with CD8βR CTL, but also prevented their maturation (Fig. S8D, F). Importantly, immature DC are pro-tumorigenic, whereas mature DC are antitumorigenic and induce endogenous, tumoricidal T cell responses.

In brief, transfer of CD8βR CTL caused extensive infiltration of endogenous CD8+ T cells into the tumor, including melanoma antigen-specific CD8+ T cells, which over time expanded in the tumor-draining lymph nodes and tumor environment (Figs. 5J, K, 6G, S4A). Supported by mature APC and Th1 cells and unimpeded by tumor-associated immunosuppression, these CTL eliminated the tumor long after the CD8βR CTL perished (Figs. 4B–D, S6C, S7A, B). Conversely, transfer of WT CTL provoked an amplification of tumor-associated immunosuppression, as manifest by increased Treg, Th17, MDSC, immature DC and PD-1high CD8+ T cells that barely expanded over time and failed to control tumor growth (Figs. 4B–D, S5–N, S7C, D). What is the potential of these findings to advance immunotherapy of cancer by adoptive transfer of tumor-specific CD8+ T cells?

CD8βR CTL upon encountering tumor cells produce very high amounts of IFNγ, which in turn induce high CXCL9 (and CXCL10) production and together are important for reprogramming the tumor microenvironment and for the generation of endogenous tumoricidal cancer-specific T cell immunity. To translate this, tumor-specific CTL could be transduced with IFNγ, which has been shown to afford high IFNγ production selectively upon CTL activation. Alternatively, high IFNγ production could be obtained by transducing tumor-specific CD8+ T cells with an active form of the Signal Transducer and Activator of Transcription 5 (STAT5). Given the crucial roles of CXCR3 receptor binding chemokines in angiostatic and in attracting activated T cells into tumors, it may be necessary to additionally transduce tumor-specific CTL with the CXCL9 (and CXCL10) genes. To mimic the high FasL expression of CD8βR CTL could by one could transduce cancer-specific CD8+ T cells with FasL. Although transport to the cell surface of FasL is specifically induced by TCR signaling (19–21), it may be considered to enforce high FasL expression by means of CAR and/or to include an inducible caspase 9-suicide gene.

It is interesting to note that our results are reminiscent to those reported for IL-12 gene therapies, i.e. reduction of tumor-associated MDSC and Treg, emergence of mature DC and induction of endogenous, tumoricidal immunity. Indeed IL-12 is known to induce IFNγ production by T and NK cells, to promote DC maturation and Fas/ FasL-dependent cytotoxicity. We suggest that the described strategies provide new immunotherapeutic modalities, namely an alternative to IL-12 gene therapy, the use of which in humans is limited by high toxicity.

Materials and methods

Mice and CTL under study

Six-week-old CD45.1+ B6.SJL and CD45.2+ P14 TCRβ chain transgenic (P14β) wild type and CD8βKO mice were bred in the Institutes animal facility. Lentiviral gene transfers were performed as described in Supplementary Methods and the CD45.2+ transduced CD8+ T cells (1 × 105) injected i.v. in CD45.1+ B6.SJL mice and these 2 d later infected with 200 PFU LCMV Armstrong and 8 d later their splenocytes isolated and analyzed or FACS sorted using reversible D5/GP33 NTAmers (TCMetryx) and anti-CD45.1 antibody to gate out the endogenous CD8+ T cells.

Tumor control experiments

B6.SJL mice were injected s.c. in the right flank with 105 B16 and/or the left flank with 107 B16-GP33 tumor cells. Mice with palpable tumors were injected i.v. with 1 × 106 of sorted D5/GP33-specific CTL and tumor volumes measured every third day with a caliper. Mice with tumors with diameters >124 mm were euthanized. In some experiments, B6.SJL mice were injected i.v. with 105 B16-GP33 tumor cells and 3 d later i.v. with 1 × 105 sorted CTL. After 21 d, the lungs were photographed using a stereomicroscope Leica M205FA and tumors enumerated using ImageJ software (NIH). In some experiments,
the tumors were excised and single cell suspensions analyzed by flow cytometry upon staining with the tetramers D\textsuperscript{b}/GP33\textsuperscript{25-33}, K\textsuperscript{b}/Trp2\textsuperscript{2180-2188}, D\textsuperscript{b}/GP100\textsuperscript{25-33} (TCMetricx). The Cantonal Veterinary Office, Lausanne, Switzerland approved all protocols. The percentage of lung surface invaded by metastatic nodules was analyzed using NIH Image J software. Briefly, lung photographs were converted in gray scale; metastatic nodules and healthy lung tissue were defined using the threshold color parameter and the respective area measured. In some experiments, the tumor bearing hosts were injected i. p. with 0.5 mg neutralizing antibodies specific for IFN\textgamma (clone XMG1.2) or CXCR3 (clone 173)(BioXCell, West Lebanon, NH) 6 h before adoptive transfer of CD8\textbeta\textbeta CTL.

Immunofluorescence labeling and microscopy were performed as described in Supplementary Methods.

Flow cytometry

Intracellular staining was performed using a cytotoxicytoperm kit from BD PharMingen. Intracellular Ca\textsuperscript{2+} measurements were performed on flow cytometry using indo-1 as Ca\textsuperscript{2+} fluorescence indicator as described previously.\textsuperscript{19} For CD107a expression, splenocytes from LCMV infected mice were stimulated with GP33 peptide in serum-free DMEM for 4 h at 37\textdegree C. For FasL intracellular staining, the cells were incubated with unlabeled anti-FasL mAb before intracellular staining. The fixed and permeabilized cells were stained antibodies specific for the respective molecules (see Supplementary Methods) and analyzed on a FACS SORP LSR II flow cytometer (BD).

Cellular assays

Specific lysis was assessed by \textsuperscript{51}Cr release essentially as described.\textsuperscript{18,19} In brief, B16, B16-GP33 tumor or D\textsuperscript{b}/PB15 cells were loaded 1 h at 37\textdegree C with \textsuperscript{51}Cr and incubated with graded concentrations of GP33 peptide, washed and incubated with CTL (E/T = 1/5 or as indicated). After 6 h, the released Cr in supernatant was counted and the specific lysis calculated. To assess the apoptosis of CD8\beta\beta or WT CTL, they were labeled with 0.25 \textmu M CFSE (CFSE\textsuperscript{low}) and incubated at different ratios of \textgamma-irradiated (30 Gy) B16-GP33 melanoma cells, pre-labeled with 2.5 \textmu M CFSE (CFSE\textsuperscript{high}). The numbers of CTL and B16-GP33 cells were enumerated by flow cytometry before and after 12 h of incubation. The apoptosis of CTL was calculated as follows: 100 - [(CFSE\textsuperscript{low}_{initial} - CFSE\textsuperscript{low}_{12h})/CFSE\textsuperscript{low}_{initial}] \times 100.

Cytokine detection

CD8\beta\beta and WT CTL were incubated with B16-GP33 cells (ratio 1:1) in presence of GolgiStop (1 \textmu g/mL)(BD). After 6 h, the cells were stained intracellularly for CCL-2, CCL-3, CXCL 9, IL-10 and TGF\textbeta gated on CD45.2\textsuperscript{+}, CD8\textsuperscript{+} T cells or B16-GP33 tumor cells to determine the percent of positive cells by flow cytometry. In some experiment, 15 d after the transfer of CD8\beta\beta or CD8\beta\beta/WT CTL into tumor bearing mice, the blood sera were collected and analyzed the content of CCL-5, CCL-3, CXCL10, CCL-4, CCL-2 chemokines and IL-17, IL-4 cytokines by using a Quantikine ELISA kit (R&D Systems).

Statistical analysis

All statistical values, i.e., average values, SD and significance differences were calculated using the GraphPad Prism software. Statistical significance between the groups was determined by using the Mann Whitney test. p < 0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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