TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance

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Summary

Tumors escape from immune surveillance by producing the immunosuppressive cytokine TGF-β. However, the mechanism by which TGF-β inhibits T cell-mediated tumor clearance in vivo is unknown. We demonstrate that TGF-β acts on cytotoxic T lymphocytes (CTLs) to specifically inhibit the expression of five cytolytic gene products—namely, perforin, granzyme A, granzyme B, Fas ligand, and interferon γ—which are collectively responsible for CTL-mediated tumor cytotoxicity. Repression of *granzyme B* and *interferon*-γ involves binding of TGF-β-activated Smad and ATF1 transcription factors to their promoter regions, indicating direct and selective regulation by the TGF-β/Smad pathway. Neutralization of systemic TGF-β in mice enables tumor clearance with restoration of cytotoxic gene expression in antigen-specific CTLs in vivo. We suggest that TGF-β suppresses CTL function in vivo through an anticytotoxic program of transcriptional repression.

Introduction

Malfunctions of the cytokine TGF-β contribute to three aspects of tumor progression, namely, loss of growth inhibitory control, gain of metastagenicity, and evasion of immune surveillance (reviewed in Akhurst and Derynck [2001], Derynck et al. [2001], Siegel and Massagué [2003]). During tumor progression, cancer cells frequently acquire resistance to the profound growth-inhibitory action of TGF-β. This may occur either by mutations that inactivate the TGF-β receptors or the Smad signal transducers, or by selective loss of downstream cytostatic, differentiative or apoptotic gene responses. In the latter case, the remaining TGF-β gene responses may foster invasion and metastasis. But in all cases, a loss of growth inhibitory control may allow tumor cells to use TGF-β for evasion of immune surveillance.

TGF-β has sweeping inhibitory effects on the immune system, negatively affecting many immune cell types and functions (Gorelik and Flavell, 2002; Letterio, 2000). TGF-β1-deficient mice present spontaneously activated T cells and die of massive inflammation in vital organs (Diebold et al., 1995). Similarly, Smad3-deficient mice develop spontaneously activated T cells that are resistant to TGF-β-mediated growth inhibition (McKarns et al., 2004; Yang et al., 1999). TGF-β controls T cell homeostasis by directly inhibiting both T cell proliferation and activation and by also inhibiting presentation of antigens on antigen-presenting cells (reviewed in Gorelik and Flavell [2002]).

Of all the mechanisms employed by tumor cells to avoid clearance, production or activation of TGF-β is considered one of the most potent (Wojtowicz-Praga, 2003). This has been demonstrated in various mouse tumor models (Torre-Amione et al., 1990; Won et al., 1999). When challenged with TGF-β-producing tumors, mice whose T cells have been made resistant to TGF-β by expression of a dominant-negative TGF-β receptor transgene are able to mount an immune response, eliminate the tumor burden, and survive (Gorelik and Flavell, 2000, 2001). Adoptive transfer experiments have demonstrated that the CD8+ T cell compartment is centrally responsible for this tumor clearance process (Gorelik and Flavell, 2001). Inhibition of TGF-β signaling in the CD8+ T cell compartment may therefore suffice to restore tumor immunity. However, little is known about the molecular mechanisms mediating TGF-β suppression of antitumor immunity by T cells.

Activated CTLs, which are predominantly responsible for antigen-specific clearance of tumor cells, typically utilize two major contact-dependent pathways to kill target cells (reviewed in Russell and Ley [2002]). One is the granule exocytosis pathway. When an activated CTL recognizes a tumor cell, the membrane-pore-forming protein, perforin, mediates delivery of the apoptosis-inducing proteases Granzyme A (GzmA) and/or

SIGNIFICANCE

The TGF-β pathway promotes cancer progression by concomitantly enhancing tumor metastases while inhibiting the host immune response. While much emphasis has been placed on understanding the paradoxical impact of TGF-β on the tumor itself, relatively little is known about the molecular effects of TGF-β on the immune response in vivo. Here we demonstrate that systemic neutralization of TGF-β activity in mice restores expression of a distinct cytotoxic gene program in CD8+ T cells, which promotes antigen-specific tumor clearance in vivo. Current cancer therapies are designed to target the TGF-β signaling pathway primarily in the tumor. We propose that therapies that also counteract the immunosuppressive effects of TGF-β and incorporate restoration of the host immune response may help achieve tumor eradication.

Granzyme B (GzmB) into the target cell. The mechanisms of GzmA- and GzmB-induced apoptosis, as defined by cleavage of their respective intracellular substrates, are quite distinct (Lieberman and Fan, 2003; Trapani and Smyth, 2002). The second contact-dependent mechanism, the Fas-Fas ligand (FasL) pathway, activates target cell death via cytochrome c release and activation of caspases (Russell and Ley, 2002). Additionally, soluble mediators including TNFα and interferon γ (IFNγ) are secreted by CTLs to induce target cell cytotoxicity.

TGF-β signaling involves its binding to a serine/threonine kinase receptor complex formed by receptor types I and II, followed by receptor-mediated phosphorylation of Smad2 and Smad3 (reviewed in Shi and Massagué [2003]; Massagué et al. [2000]). Thus activated, Smad2 and -3 then bind to Smad4. The Smad2/3-Smad4 complex associates with other DNA binding proteins, forming different complexes of defined target gene specificity and transcriptional activation or repression activity. For example, a TGF-β cytostatic gene response program has been delineated in epithelial cells, and the cofactors that enable Smad complexes to target and regulate these genes have been identified (Chen et al., 2002; Kang et al., 2003; Seoane et al., 2002; Siegel et al., 2003).

The molecular mechanisms suppressing T cell activation in response to TGF-β have yet to be elucidated. To address this problem, we employed a mouse tumor model in which neutralization of systemic TGF-β activity results in tumor clearance. Using transcriptomic profiling, we identified a striking suppression by TGF-β of factors that constitute the CTL cytotoxic program—perforin, GzmA, GzmB, FasL, and IFNγ. At least two of these genes, *GzmB* and *IFNγ*, are directly recognized by Smads and ATF1 in response to TGF-β, indicating that the CTL cytotoxic program is directly targeted by the Smad pathway. Furthermore, we demonstrate that neutralization of TGF-β restores expression of these genes in tumor-specific CD8+ T cells in vivo, leading to tumor clearance. These results suggest that TGF-β suppresses CD8+ T cell cytotoxicity in vivo through repression of a distinct program of cytotoxic gene responses.

Results

Neutralization of systemic TGF-β restores T cell cytotoxicity and tumor clearance in vivo

Various tumor models have been employed to establish the role of TGF-β in promoting tumor growth in vivo. One such tumor model is the EL4 thymoma cells, which secrete TGF-β1 and may additionally coerce the production or activation of TGF-β from host tissue sources (Gorelik and Flavell, 2001; Won et al., 1999). Intraperitoneal injection of EL4 thymoma cells into mice leads to tumor burden within 7–10 days and death in approximately 3 weeks. Previous studies have indicated that systemic neutralization of TGF-β in vivo or inhibition of TGF-β signaling specifically in the T cell compartment restores tumor cell clearance (Gorelik and Flavell, 2001; Won et al., 1999). Using two approaches to abrogate the immunosuppressive activity of TGF-β, we analyzed this tumor model to determine how TGF-β mediates immune evasion in vivo. First, to determine whether the tumor cells were the major source of TGF-β, we generated an EL4-derivative cell line (EL4-shTGFβ1) that stably expresses a short hairpin RNAi targeting TGF-β1. Expression of TGF-β1 in EL4-shTGFβ1 cells is decreased by 85% compared to parental EL4 (Figure 1A). Expression of TGF-β2 and TGF-β3 was not detectable in EL4 cells (data not shown). Second, EL4 cells were infected with the empty vector (EL4-pRS cells) or with a retroviral construct encoding soluble TGF-β type II receptor (TβR-II) extracellular domain to neutralize tumor-derived and surrounding sources of TGF-β (Figure 1A). All three cell lines were injected into cohorts of syngeneic mice, and tumor development and survival were monitored. Mice injected with EL4-pRS developed significant tumor burdens, with 80% of the mice having to be sacrificed between 16 and 20 days (Figure 1B). While there was a delay in tumor development, 75% of the mice injected with EL4-shTGF-β1 tumors ultimately succumbed to the tumor burden, (p value = 1.0). In contrast, only 20% of the mice injected with EL4 cells expressing soluble TβR-II died (EL4-soluble TβR-II; p value = 0.008; Figure 1B). Therefore, neutralization of systemic TGF-β, but not reduction of tumor-derived TGF-β, could restore tumor-free survival in mice.

To determine the effect of these TGF-β-blocking strategies on T cell-mediated tumor lysis, CD8+ T cells were assessed for cytotoxic activity against EL4 tumor cells, using a standard ^{51}Cr-release assay (Heusel et al., 1994). When cultured with labeled EL4 tumor cells directly following purification, only CD8+ T cells derived from EL4-soluble TβR-II-injected mice were able to kill EL4 tumor cell targets, in a dose-dependent manner (Figure 1C). These CD8+ T cells were capable of recognizing and killing both EL4 and EL4-soluble TβR-II tumor cells in vitro, indicating that the immune response was directed against the tumor cell itself and not the soluble receptor (Figure 1D). Even at the highest T cell-to-tumor ratio (100:1), CD8+ T cells derived from mice injected with either EL4-pRS or EL4-shTGF-β1 tumor cells were unable to kill target cells. Characterization of the CD8+ T cells by flow cytometry did not show significant differences in CD8+ T cell number, cell size, or expression of the activation marker CD69 among the three cohorts of mice (data not shown). In addition, TGF-β treatment of EL4 tumor cells in vitro did not result in tumor cell growth arrest or apoptosis, indicating that the effects of TGF-β in vivo are most likely not on the tumor itself (data not shown). Therefore, in this model system, systemic neutralization of TGF-β in vivo is required for specifically restoring T cell-mediated cytotoxicity, leading to tumor clearance.

TGF-β represses a cytotoxic gene program in T lymphocytes

To identify TGF-β-target genes in T cells that would favor tumor evasion in vivo, we performed transcriptomic profiling of freshly isolated primary mouse T lymphocytes activated ex vivo. T cells become activated upon antigen recognition and signaling through the T cell receptor-CD3 complex, with concomitant CD28 costimulation. To mimic T cell receptor engagement in vitro, T cells were stimulated with anti-CD3 plus anti-CD28 antibodies, in the presence or absence of TGF-β. RNA from these cells was used to probe Affymetrix U74Av2 mouse genome microarrays, which had 12,422 probe sets containing 9198 unique genes and 430 ESTs. The expression of approximately 69 known genes and 18 ESTs was increased, and 35 known genes and four ESTs decreased (>2.0 fold change, p = 0.001) by TGF-β under these conditions (Figure 2A and see

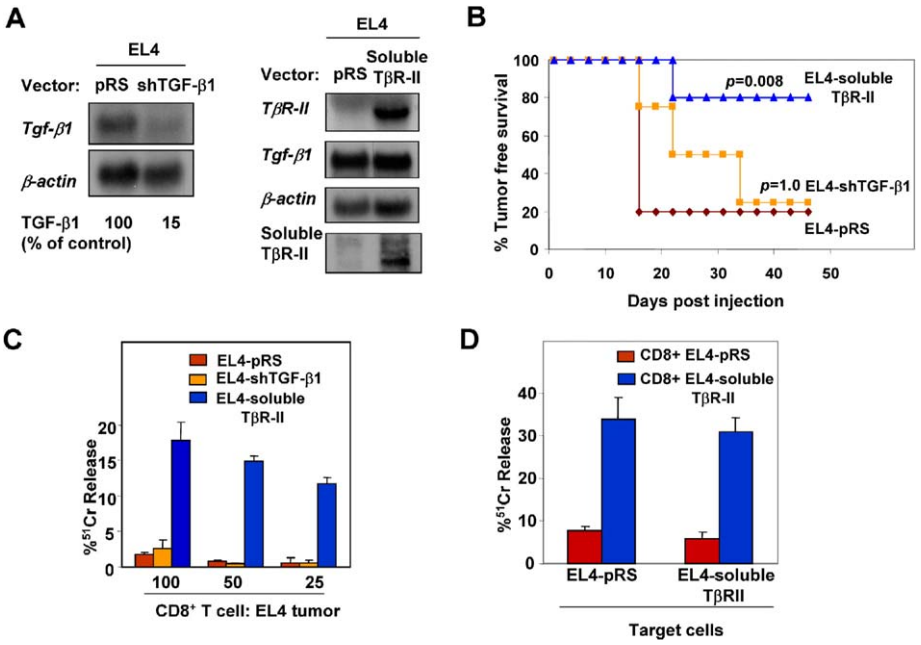

Figure 1. Neutralization of systemic TGF-β in vivo restores T cell cytotoxicity

A: EL4 tumor cells were infected with either empty retroviral vector (pRS), with retroviruses expressing short-hairpin RNAs against TGF-β1 (shTGF-β1), or encoding a soluble form of TβR-II. Northern analysis was performed for *TβR-II* and *Tgf-β1*. Soluble TβR-II expression was detected in cell culture supernatants by immunoblot.

B: Syngeneic mice were injected i.p. with 2.5 × 10^6 EL4-pRS, EL4-shTGF-β1, or EL4-soluble TβR-II cells and observed for tumor growth and survival. P values were obtained using Fisher's exact test on two independent experiments.

C and D: On day 10 postinoculation, CD8+ T cells were purified from each cohort of EL4-injected mice and cultured for 3 hr with ^{51}Cr-labeled EL4 (**C**) or EL4-pRS and EL4-soluble TβR-II tumor cells (**D**) at an E:T ratio of 100.

Each point was performed in triplicate, with the mean percent ^{51}Cr release and standard deviation shown here.

Table S1 in the Supplemental Data available with this article online). These responses included induction of the cell adhesion receptor *integrin αE* and the TGF-β-negative feedback genes *smad7* and *ski*, and downregulation of the growth-promoting transcription factor *c-myc*. These TGF-β responses are typical in other cell types and serve here as a reference. Five additional downregulated genes, *perforin*, *granzyme A* (*GzmA*), *granzyme B* (*GzmB*), *interferon-γ* (*IFNγ*), and *Fas ligand* (*FasL*) (highlighted in yellow, Figure 2A), were of particular interest because all are mediators of CTL-mediated cytotoxicity and contribute to tumor clearance (Russell and Ley, 2002). While the perforin-Gzm pathway is the major effector arm of CTL-mediated cytotoxicity, the Fas-FasL pathway and IFNγ secretion have also been implicated in tumor regression (Lee and Ferguson, 2003; Shankaran et al., 2001; Street et al., 2001; Trapani and Smyth, 2002). Repression of these genes would explain the suppressive effects of TGF-β specifically on CTLs.

Upon T cell stimulation, expression of these five genes indicates differentiation of the naive CD8+ T cell population into activated CTLs (Schluns and Leofrancois, 2003). To determine whether TGF-β inhibited overall CD8+ T cell activation, we assessed expression of various cell surface receptors and activation markers. Using FACS analysis, we observed that TGF-β did not affect the proportion of primary T cells expressing the TCR CD3ϵ chain or the coreceptors CD4 and CD8 (Figure 2B). More importantly, TGF-β treatment did not alter expression of the activation markers, CD69 or CD44^{hi}, indicating that the CD8+ T cells were comparably activated (summarized in Figure 2B). Loss of CD62L, a naive T cell marker that is downregulated upon T cell activation, was also not altered by TGF-β treatment. While there was a slight decrease in expression of the IL-2 receptor α chain (CD25) expression, this suggests an effect on T cell proliferation rather than T cell activation. Therefore, the decrease in cytotoxic gene expression was not due to selective depletion of CD8+ cells from the T cell population or inhibition of overall CD8+ T cell activation.

Inhibition of *perforin*, *GzmA*, *GzmB*, *IFNγ*, and *FasL* expression by TGF-β at the mRNA level was verified in purified CD8+ T cells (Figure 2C). We also analyzed protein expression by intracellular FACS staining and confirmed that TGF-β inhibits expression of all five genes to varying degrees (Figure 2D). TGF-β decreased both the proportion of CD8+ T cells expressing GzmB and the level of GzmB expression (Figure 2D). The number of CD8+ T cells expressing high levels of IFNγ was also decreased, while the number of CD4+ T cells that expressed IFNγ at all was low (Figure 2D and data not shown), which corresponded to the levels of secreted IFNγ (Figure 2E). Activation of T cells using anti-CD3 and anti-CD28 antibodies leads to relatively low levels of GzmA, perforin, and FasL, compared to levels of GzmB and IFNγ. However, TGF-β clearly inhibited the expression of perforin, GzmA, and FasL in these cells (Figure 2D). Thus, five genes constituting the cytotoxic gene program in CD8+ T cells are selectively repressed by TGF-β.

We next assessed the ability of TGF-β to inhibit the expression of these genes in response to intact tumor stimulation of T cells in vitro. Spleen cells were cultured with the irradiated tumor cell line, P815, in mixed lymphocyte reactions, in the presence or absence of TGF-β, to generate tumor-specific CTLs (MLR-CTLs) (Heusel et al., 1994). Following activation with P815 tumor cells, expression of *Perforin*, *GzmA*, *GzmB*, and *FasL*, but not *IFNγ*, could be detected by Northern analysis, and continuous treatment with TGF-β inhibited the expression of these genes (Figure 3A). Protein immunoblotting analysis confirmed that perforin, GzmA, and GzmB production was repressed in these cells (Figure 3A). Compared to antibody-mediated activation of primary T cells, the activation mediated by P815 tumor cells causes a robust induction of GzmA, comparable to GzmB, but TGF-β inhibited the expression of both

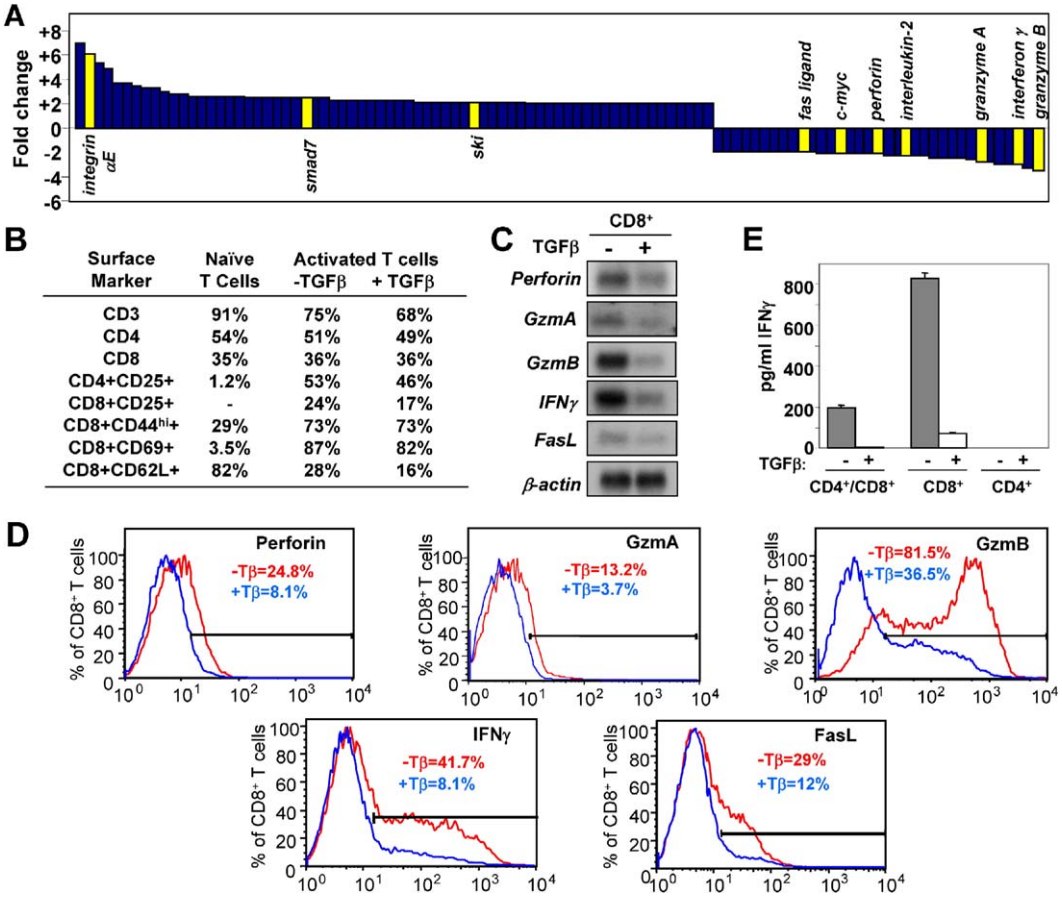

Figure 2. Identification of the TGF-β-regulated cytotoxic gene program in primary T lymphocytes

A: CD4+/CD8+ T cells were purified from mouse spleens and activated with 0.25 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 antibodies in the presence or absence of 100 pM TGF-β. Transcriptomic profiling was performed with the mouse U74Av2 Affymetrix microarrays. The plot shows the fold change gene expression +TGF-β/−TGF-β. Genes of interest are labeled in yellow (see also Table S1).

B: Primary T cells were activated, as in (**A**), for 48 hr and analyzed for expression of cell surface markers cells by flow cytometry.

C: Primary CD8+ T cells were activated as in (**A**), and Northern analysis was performed with the indicated probes.

D: Primary T cells were activated as in (**A**) for 48 hr and stained for the surface receptors CD4 and CD8, followed by intracellular staining with the indicated fluorescent antibodies. CD8+ T cells were analyzed for expression of Perforin, GzmA, GzmB, IFNγ, or FasL.

E: CD4+, CD8+, or bulk T cells were purified and activated as in (**A**) for 48 hr. Supernatants were analyzed in triplicate for IFNγ secretion by ELISA.

granzymes. The inhibitory effect of TGF-β on *FasL* expression was reproducible but small (Figure 3A).

MLR-CTLs were then assayed for their ability to kill tumor cells in vitro. TGF-β inhibited the proliferation of MLR-CTLs by 40%–50% (data not shown). However, even after normalization of effector-to-tumor cell ratios, MLR-CTLs treated with TGF-β were approximately 3-fold less effective in rapid (3 hr) tumor cell lysis, as measured by ^{51}Cr release (Figure 3B, top left panel). MLR-CTLs can also induce DNA fragmentation (^{125}IdUR release) in the target cell, which was also found to be abrogated by TGF-β, compared to unmodified MLR-CTLs (Figure 3B, top right panel). Even at later time points (6 hr), we found that TGF-β-treated MLR-CTLs were still unable to induce equivalent ^{51}Cr or ^{125}IdUR release from tumor cell targets (Figure 3B, bottom). Collectively, these observations indicate that in vitro TGF-β inhibits the ability of CTLs to kill tumor cells via the GzmA-perforin, GzmB-perforin, and Fas-FasL pathways, correlating with the ability of TGF-β to inhibit expression of these genes.

TGF-β represses the cytotoxic gene program independently of growth inhibition

Another significant effect of TGF-β on T cells is the inhibition of interleukin-2 (IL-2) expression (McKarns et al., 2004), as confirmed, both at the mRNA level (Figure 2A; Northern analysis data not shown) and the secreted protein level (Figure 4A). This effect correlates with T cell growth arrest (Figure 4B). As IL-2 is induced upon T cell activation to stimulate T cell proliferation, we wondered whether the decrease in the overall cytotoxic gene expression by TGF-β was secondary to inhibition of T cell proliferation. To address this question, we used recombinant IL-2 (rIL-2) as the mitogenic stimulus in primary T cells, thus bypassing the need for T cell receptor activation and autocrine IL-2 production. Since naive T cells only express the low-affinity IL-2 receptor β and γ chains but not the high-affinity α chain, we chose a range of rIL-2 concentrations that would achieve proliferation comparable to T cells stimulated through their T cell receptor. TGF-β only partially inhibited rIL-2-driven T cell proliferation (Figure 4C), whereas it profoundly inhibited

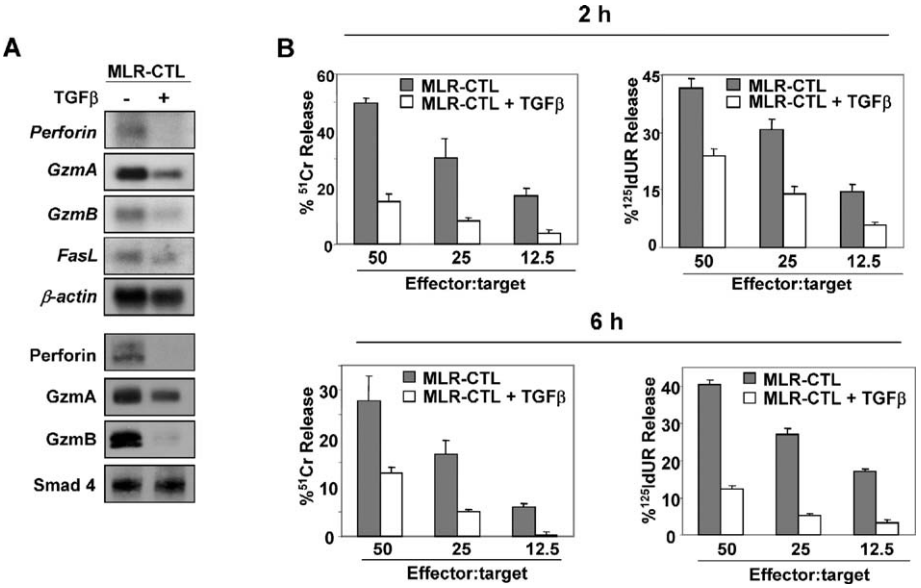

Figure 3. TGF-β represses cytotoxic gene expression to inhibit CTL-induced tumor cell cytotoxicity in vitro

A: Primary MLR-CTLs were generated by stimulation with irradiated P815 tumor cells for 5 days, with or without TGF-β, and Northern and immunoblot analyses were performed for the indicated genes.

B: Primary MLR-CTLs, minus and plus TGF-β, were incubated with ^{51}Cr or ^{125}IdUR-labeled P815 target cells for 2 or 6 hr at various effector-to-target cell ratios. ^{125}IdUR release is a measurement of DNA fragmentation, and ^{51}Cr release is a measurement of tumor cell lysis.

Each point was performed in triplicate, with the mean percent ^{51}Cr and percent ^{125}IdUR release and their respective standard deviations shown here.

the expression of *Perforin*, *GzmA*, *GzmB*, and *IFNγ* in these cells (Figure 4D).

To better separate the antimitogenic and anticytotoxic effects of TGF-β, we analyzed these effects in the IL-2-dependent, CD8+ T cell line CTLL-2. These cells express high levels of CD25 and the activation marker CD69, indicating that the cells are in an activated state and can respond readily to IL-2 stimulation. Using multiple rIL-2 concentrations, we found that TGF-β inhibits CTLL-2 proliferation after 24 hr of concurrent IL-2 and TGF-β treatment (Figure 4E). In contrast, expression of both *perforin* and *GzmB* is rapidly repressed within 4 hr of TGF-β treatment (Figure 4F). Repression of *GzmA* by TGF-β in these cells was not observed within this time frame, and IFNγ expression was not detectable (data not shown). Thus, while IL-2 is critical for both T cell proliferation and cytotoxic gene expression, TGF-β can directly and rapidly inhibit expression of the cytotoxic gene program, downstream of IL-2.

Direct Smad-ATF1 binding to *GzmB* and *IFNγ* promoter regions

Having identified multiple cytotoxic genes that are critical TGF-β targets, we next wanted to determine whether these genes are direct targets of the TGF-β-activated Smad pathway. Focusing on TGF-β-mediated repression of GzmB, we cloned 3 kb of the mouse *GzmB* upstream region in front of a *luciferase* reporter gene and performed reporter assays in CTLL-2 cells (Figure 5A). Promoter deletion analysis mapped the TGF-β-responsive region at position –885/–794 (Figures 5A and 5B). Inspection of this 92 bp region revealed binding sites for transcription factors AML1α, ATF1/CREB, and Oct1/GATA1 in proximity to potential Smad binding elements (Figure 5B). Mutational analysis revealed that the ATF1/CREB site was required for TGF-β-induced repression in this assay system (Figure 5C).

To determine whether GzmB repression required new protein synthesis, CTLL-2 cells were pretreated with cycloheximide (CHX) prior to TGF-β treatment. In the presence of CHX, TGF-β was no longer able to repress GzmB, unlike c-Myc repression, which does not require new protein synthesis (Figure 5D; Chen et al., 2002). When we analyzed ATF1 and CREB expression, we found that both genes were induced by TGF-β, even in the presence of CHX (Figure 5D). The ability of TGF-β to induce expression of transcription factors that subsequently function as Smad partners to regulate other target genes is a phenomenon that has been previously described in other TGF-β-regulated processes (Massagué et al., 2005). In epithelial cells, TGF-β elevates expression of another ATF family member, ATF3, in a first-wave response, which then cooperates with the Smads to repress *Id1* expression (Kang et al., 2003).

To establish that a TGF-β-activated Smad complex binds directly to the *GzmB* promoter in vivo, we performed chromatin immunoprecipitation (ChIP) analyses in primary T cells activated in the presence or absence of TGF-β (Figure 5E). TGF-β induced the binding of Smad2/3 over the –930/–585 region of the *GzmB* promoter in primary T cells (Figure 5F). In addition, both ATF1 and CREB bound to this same region within the *GzmB* promoter in a TGF-β-inducible manner (Figure 5E).

ATF1 has also been implicated in the repression of *IFNγ* during differentiation of CD4+ Th2 cells (Penix et al., 1996). Upon examination of the *IFNγ* proximal promoter (–300/+4), we noticed two potential ATF1 binding sites near a Smad binding element (Figure 5F), suggesting that TGF-β may utilize the same mechanism to regulate expression of this cytotoxic gene. ChIP assays in primary T cells demonstrated inducible binding of ATF1 and Smad2/3, but not CREB, to the *IFNγ* promoter in response to TGF-β (Figure 5E). Binding was not detected on the β-actin promoter, which served as a negative control.

To determine the necessity of ATF1 and CREB, as well as the Smads, for TGF-β-mediated GzmB repression, we performed transient knockdown experiments using RNAi-mediated technology. CTLL-2 cells were transfected using siRNA oligonucleotides against GFP (siGFP) as a control or oligonucleotides specific for ATF1 and CREB, which demonstrated a 80% and 75% loss of expression of these proteins, respectively (Figure 5G). Treatment of cells with TGF-β demonstrated that, with re-

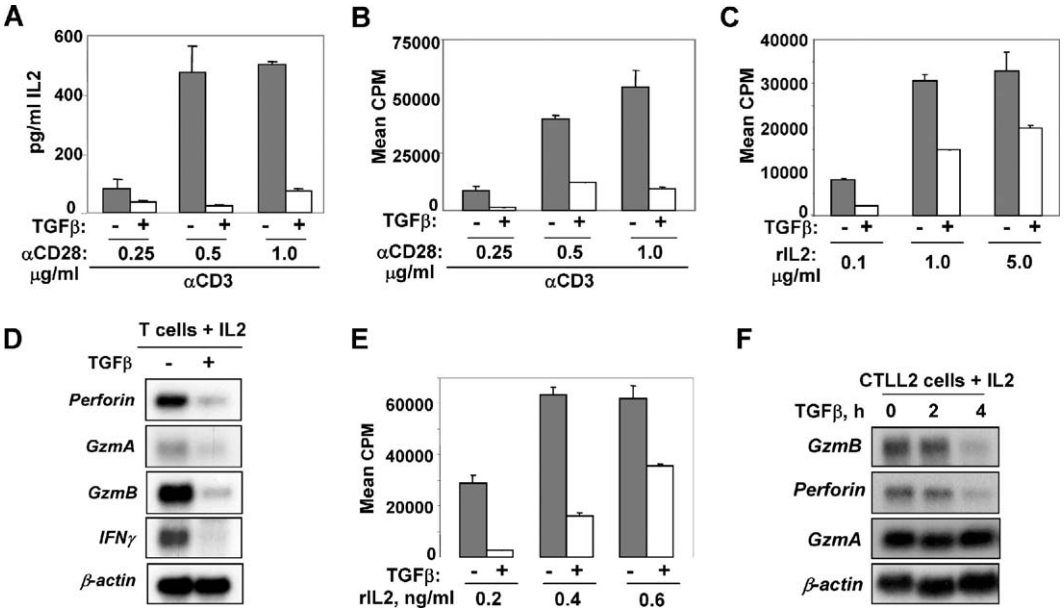

Figure 4. TGF-β directly represses cytotoxic gene expression, independent of growth inhibition

CD4+/CD8+ T cells were activated with anti-CD3 plus various concentrations of anti-CD28 antibodies. IL-2 secretion was measured by ELISA (**A**), and proliferation was measured by ^{125}IdUR incorporation (**B**). (**C**) Primary T cells were stimulated with rIL-2, in the presence of absence of TGF-β, for 48 hr, and growth inhibition was measured via ^{125}IdUR incorporation. (**D**) Northern analysis was performed for the indicated genes in primary T cells activated with 1.0 μg/ml rIL-2 for 24 hr. (**E**) CTLL-2 cells were stimulated with rIL-2, and proliferation was measured after 24 hr. (**F**) CTLL-2 cells were stimulated with 0.4 ng/ml rIL-2, and Northern analysis was performed after 2 or 4 hr of TGF-β treatment with the indicated probes.

duced levels of ATF1 and CREB, TGF-β was not as effective in repressing GzmB, compared to control transfected oligonucleotides (46% versus 20%, Figure 5G). As a control, loss of ATF1 and CREB did not affect cMyc repression (Figure 5G). Finally, we also coordinately knocked down expression of both Smad2 and Smad3 in CTLL-2 cells (approximately 75% for each, Figure 5H) and found that, in the absence of Smad2 and 3, TGF-β could not repress expression of GzmB or perforin, while loss of Smad3 alone had little effect (Figure 5H). While the detailed mechanism of *GzmB* and *IFN*γ repression by Smad, ATF1, and CREB remains to be defined, the present results provide evidence that TGF-β targets these promoters in T cells via Smad transcription factors. The direct nature of this interaction is consistent with a selective inhibition of the CTL cytotoxic program by TGF-β.

IL-2 restores TGF-β-suppressed T cell proliferation and cytotoxic gene expression

We next investigated whether we could rescue expression of the cytotoxic gene program and/or T cell proliferation following TGF-β treatment. We compared the effects of two members of the common cytokine receptor γ chain (γc) family: IL-2, because it induces cytotoxic gene expression and proliferation in naive T cells, and IL-15, because it specifically regulates CD8+ T cell proliferation and the generation of effector memory cells (Schluns and Leofrancois, 2003). Primary T cells were activated in the presence or absence of TGF-β for 36 hr and then washed and recultured in either fresh media alone (untreated), in media plus IL-2, or in media plus IL-15 for another 36 hr (Figure 6A). Although T cells were growth inhibited by TGF-β treatment (refer to Figure 3B), removal of TGF-β and reculturing cells in media alone restored equivalent proliferation levels (Figure 6B). Both IL-2 and IL-15 could restore comparable proliferation in both TGF-β-treated and -untreated cells, although IL-2 was a more potent growth stimulator (Figure 6B). IL-2 increased the proportion of CD8+ T cells by 7%–10%, while IL-15 increased it only 3%, irrespective of pretreatment with TGF-β (Figure 6C). Neither cytokine significantly altered the proportion of CD4+ T cells. IL-15 also did not stimulate IL-2 secretion under any conditions assayed here (data not shown).

We next analyzed expression of the cytotoxic effectors GzmB and IFNγ to determine whether IL-2 or IL-15 could rescue their expression in TGF-β-treated CD8+ T cells. IL-2 induced significant IFNγ expression in both activated T cell populations, despite lower basal levels in TGF-β-pretreated cells, while IL-15 did not induce IFNγ secretion (Figure 6D). The majority of activated T cells demonstrated significant GzmB expression, and reculturing with IL-2 or IL-15 had little effect (Figure 6E, left panel). In contrast, T cells activated in the presence of TGF-β had lower basal levels of GzmB expression, which could be rescued with IL-2 treatment but not with IL-15 (Figure 6E, right panel). Although only 27% of TGF-β-treated cells express GzmB, IL-2 increased the percentage to 91.2%, comparable to levels in T cells that had not received TGF-β (Figure 6E, left panel). In sum, IL-2 can rescue T cell proliferation and GzmB and IFNγ expression in T cells that were previously exposed to TGF-β, whereas IL-15 can partially restore T cell proliferation but not expression of cytotoxic genes.

TGF-β neutralization restores cytotoxic gene expression in antigen-specific T cells in vivo

We have shown that TGF-β represses a distinct cytotoxic gene program, which inhibits the overall ability of CD8+ T cells to kill

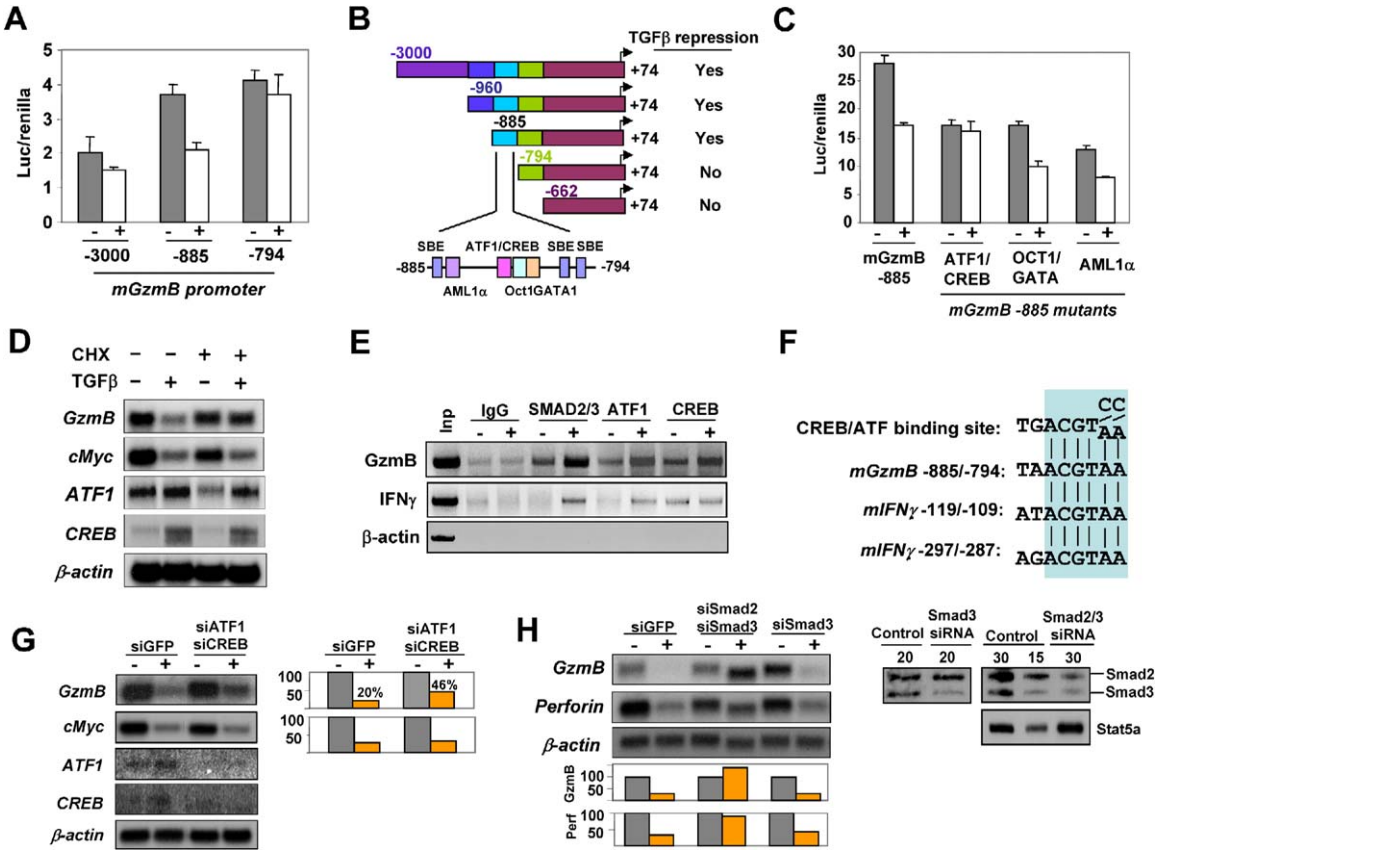

Figure 5. TGF-β-mediated repression of GzmB and IFNγ involves ATF1 and Smads 2/3 binding to both promoters in vivo

A: Full-length (3.0 kb) mouse *GzmB* promoter and sequential deletion constructs were cloned upstream of *luciferase* reporter constructs and delivered into CTLL-2 cells via nucleofection (Amaxa). Luciferase activity was measured following 20 hr of TGF-β treatment. Each point was performed in triplicate with mean Luc/renilla values plotted with their standard deviations.

B: Schematic of transcription factor binding sites within the −885/−794 *mGzmB* promoter.

C: Mutations were generated within the ATF1/CREB, Oct1/GATA1, or AML1α sites of the *GzmB* −885/+74 promoter, and luciferase assays were performed as in (**A**).

D: CTLL-2 cells were pretreated with CHX prior to treatment with TGF-β for 4 hr. Northern analysis was then performed for the indicated genes.

E: ChIPs for *GzmB*, *IFNγ*, and β-actin were performed using the indicated antibodies on primary T cells, activated in the presence or absence of TGF-β.

F: Alignment of *GzmB* and *IFNγ* promoters, showing ATF1/CREB binding sites.

G and H: Knockdown of ATF1 and CREB (**G**) or Smad2 and Smad3 (**H**) was performed using transient transfection of siRNA oligonucleotides into CTLL-2 cells. siRNA oligonucleotides against GFP were used as control. Posttransfection (48 hr), cells were treated with TGF-β for 3 hr and RNA was harvested for Northern analysis. Depletion of *ATF1* and *CREB* mRNA levels in (**G**) is shown by Northern blot analysis. Depletion of Smad3 alone or Smad2 and Smad3 is shown by Western blot analysis using Stat5a as a negative control. The amount of protein cell lysate (μg) is indicated.

tumor cells in vitro. However, we wanted to determine if TGF-β affects expression of these cytotoxic effectors in vivo. Since in vivo T cells respond to unique antigens displayed on the tumor cell surface, we employed an antigen-specific tumor model to identify which genes are restored in antigen-specific CD8+ T cells by neutralization of TGF-β. We used the EL4 thymoma subline (E.G7), which has been engineered to express the ovalbumin antigen (OVA). SIINFEKL has been identified as the dominant OVA peptide presented in the context of MHC class I H-2K^b on the tumor cell surface (Gorelik and Flavell, 2001; Makki et al., 2002). The majority of T cells reacting to EL4-OVA tumor cells will recognize SIINFEKL as the antigenic tumor peptide. To determine whether neutralization of TGF-β alters the quantity and/or quality of antigen-reactive T cells in vivo, we engineered EL4-OVA cells to express the soluble TβR-II trap (Figure 7A). CD8+ T cells that recognize this tumor antigen can be identified using flow cytotmetry plus fluorescently labeled tetramers of recombinant H-2K^b molecules loaded with the SIINFEKL peptide (Altman et al., 1996; Appay and Rowland-Jones, 2002). Once identified, these T cells could be further analyzed for cytotoxic effector expression using intracellular staining (as in Figure 2). Mice were injected with either EL4-OVA cells, EL4-OVA-soluble TβR-II cells, or the original EL4-pRS cells as a negative control since they do not express the OVA antigen. Spleen cells harvested 10 days later demonstrated that the percentages of CD4+ and CD8+ T cells were equivalent in mice injected with the EL4-OVA cell lines, indicating that there was no skewing of the T cell populations in vivo (Figure 7B). In addition, the activation markers CD69 and CD44 were comparably expressed, and downregulation of CD62L was observed in the spleens of all three groups as compared to naive CD8+ T cells (Figures 7C and 7D and data not shown).

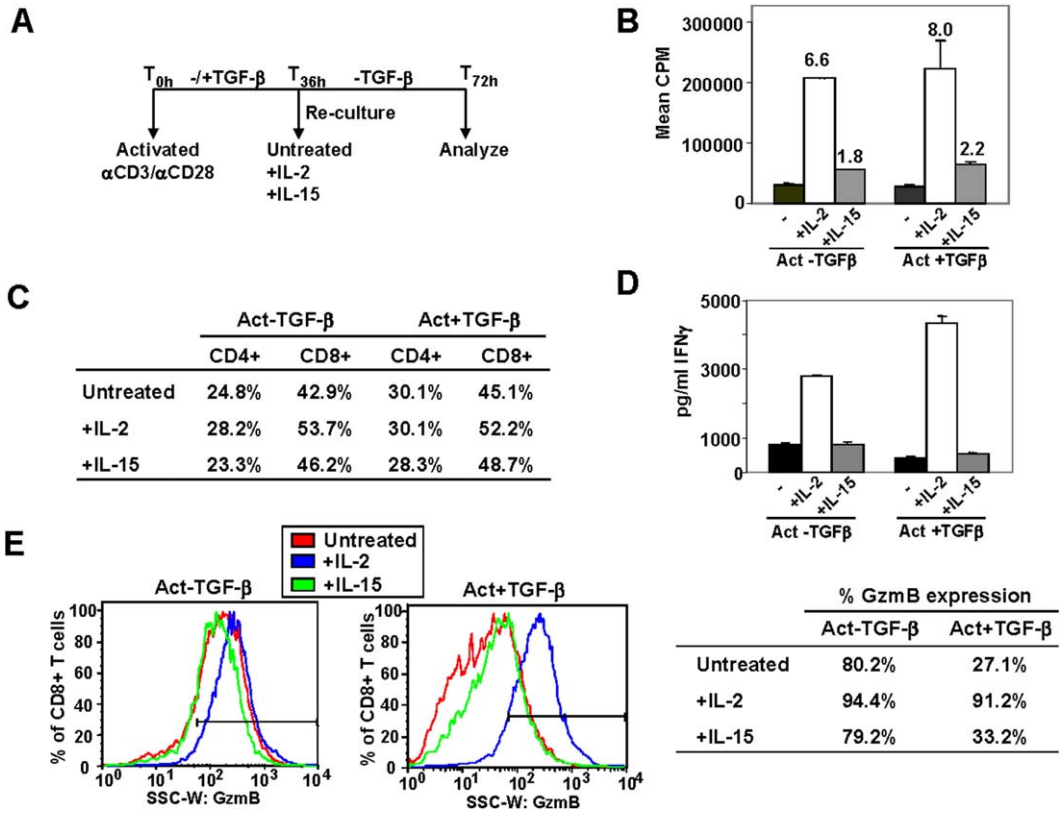

Figure 6. IL-2 rescues both T cell proliferation and cytotoxic gene expression following TGF-β treatment

A: T cells were activated in the presence or absence of TGF-β for 36 hr, washed extensively, and then recultured in media alone (untreated) plus rIL-2 or plus rIL-15 for another 36 hr.

B–E: Proliferation (^{125}IdUR incorporation) (**B**) expansion of CD4+ and CD8+ T cells (**C**), IFNγ secretion (**D**), and intracellular GzmB staining (**E**) were analyzed in untreated T cells or T cells cultured with rIL2 or rIL-15, as described in (**A**). Mean CPMs with standard deviations from triplicate samples (**B**) and mean concentrations with standard deviations from samples in triplicate (**D**) are shown here.

Since comparable numbers of T cells were activated, either directly or indirectly, by the tumor challenge, we conclude that TGF-β does not alter the activation profile of OVA-specific CD8+ T cells in vivo.

To determine whether there was an increase in the proportion of antigen-specific T cells that develop when TGF-β is neutralized in vivo, we next analyzed the proportion of CD8+, tetramer-positive T cells. Analysis of spleen cells derived from EL4-pRS-injected mice demonstrated background levels of tetramer staining (0.3%; green lines, Figure 7E). When background tetramer staining was subtracted, the proportion of tetramer-positive (i.e., antigen-specific) T cells that developed in the presence of TGF-β was very low (0.2% ± 0.04%; blue lines). This proportion is consistent with previous reports from EL4 tumor-inoculated mice (Gorelik and Flavell, 2001). In contrast, expression of soluble TβR-II in these tumors increased the number of antigen-specific T cells 4- to 5-fold compared to EL4-OVA-injected mice (1.1% ± 0.3% versus 0.2% ± 0.04%; red lines, Figure 7E). In general, the number of tetramer-positive T cells that develop in response to tumors or pathogens in vivo is approximately 1% of the population (Altman et al., 1996). Therefore, TGF-β inhibits antigen-specific T cell expansion, and neutralization of systemic TGF-β restores their development.

Based on these results, we sought to determine whether in vivo neutralization of TGF-β could restore expression of the same cytotoxic effectors that were identified as TGF-β target genes in vitro. Combining surface staining of tetramer-positive T cells with intracellular staining, we quantitated the fraction of cells expressing either perforin, GzmA, GzmB, IFNγ, or FasL. We found that neutralization of TGF-β in vivo augmented the expression of perforin, GzmA, GzmB, and IFNγ (1.9- to 3-fold increase) but not FasL, compared to mice injected with EL4-OVA tumor cells (Figures 7E and 7F). Similar results in gene expression were obtained in bulk CD8+ T cells derived from mice injected with EL4-soluble TβR-II tumor cells (data not shown). Thus, inhibition of TGF-β in vivo not only increases the proportion of T cells that can respond to the tumor but also restores the activities of the perforin-gzm pathway and IFNγ secretion for tumor clearance.

Discussion

Selective and direct inhibition of the T cell cytotoxic gene responses by TGF-β

During tumor progression, production of TGF-β leads to escape from immune surveillance in vivo (Akhurst and Derynck, 2001; Derynck et al., 2001; Siegel et al., 2003). Since identifica-

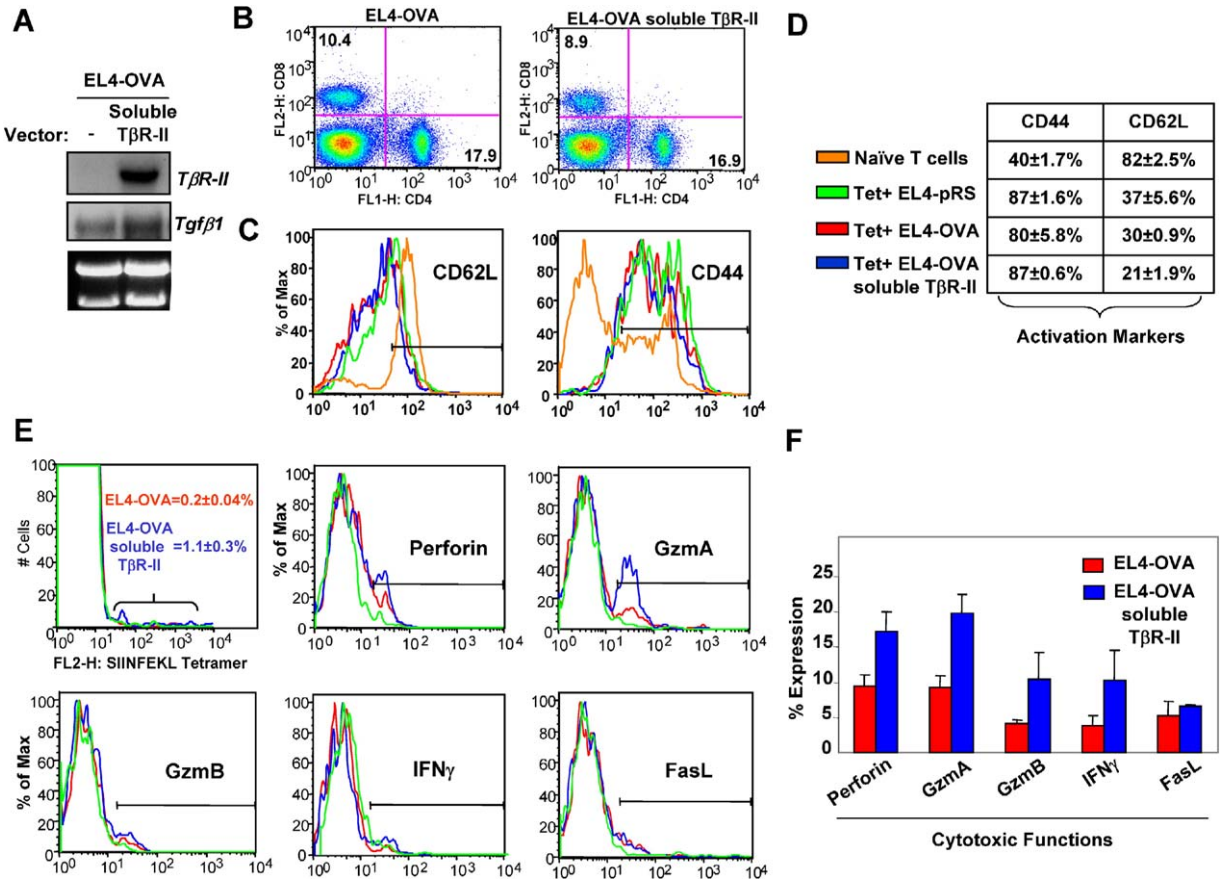

Figure 7. Neutralization of TGF-β restores expression of perforin, GzmA, GzmB, and IFNγ in antigen-specific T cells in vivo

A: Expression of TGF-β1 and soluble TβR-II in EL4-OVA tumor cells was determined by Northern analysis.

B–D: On day 10 postinjection, spleen cells were harvested from EL4-pRS (green lines), EL4-OVA (red lines), or EL4-OVA-soluble TβR-II (blue lines)-injected mice and analyzed for the number of T cells expressing CD4 or CD8 (**B**) or the activation markers CD44 and CD62L (**C**) via flow cytometry and quantitated (**D**).

E and F: Spleen cells were stained with H-2K^b SIINFEKL-specific, PE-conjugated tetramers plus CD8 antibodies and analyzed by flow cytometry. The percentages represent live, tetramer-specific, CD8+ T cells with a subtracted background of tetramer-reactive T cells from EL4-pRS-injected mice. Following tetramer staining, intracellular staining was performed using indicated antibodies and analyzed. The percentage of tetramer-specific T cells expressing perforin, GzmA, GzmB, IFNγ, or FasL is quantitated among the EL4-tumor cell lines. One representative experiment is shown. Experiments were repeated at least three times, and the mean expression level of each gene with the standard error is plotted (**F**).

tion of the relevant T cell gene responses in vivo is essential for understanding TGF-β-mediated tumor evasion, we performed a comprehensive analysis of TGF-β-responsive genes in T cells. Focusing on CD8+ T cells, we have identified the cytotoxic mediators perforin, GzmA, GzmB, IFNγ, and FasL as five critical TGF-β target genes, collectively referred to here as the cytotoxic program. The extent of the induction of these five genes upon T cell stimulation and the extent of their repression by TGF-β in our experiments varied with different in vitro and in vivo conditions. However, repression of all or even part of these genes would limit CTL-mediated tumor clearance. Regulation of some of these genes was noted in early studies of T cell lines (Genestier et al., 1999; Inge et al., 1992; Smyth et al., 1991) or as part of a general effect of TGF-β on T cell development in vitro (Bonig et al., 1999). We show that the expression of these genes is inhibited by TGF-β independently of a block in CD8+ T cell proliferation or activation, and it involves a selective inhibition of the CTL cytotoxic program both in vitro and in vivo.

We provide evidence that the effect of TGF-β on cytotoxic gene responses occurs with the direct involvement of the Smad signaling pathway. We identified a TGF-β-responsive region in the GzmB promoter, which requires an intact ATF1/CREB binding site. Strikingly similar to another ATF family member and TGF-β target gene, ATF3, we found that ATF1 and CREB are both induced by TGF-β in the presence of CHX. As in the case of TGF-β-induced ATF3 cooperating with Smads to repress *Id1* in epithelial cells (Kang et al., 2003), TGF-β-induced ATF1 and CREB may cooperate with Smads to repress *GzmB* and *IFNγ* in T cells. Such self-enabled gene responses have been described in other TGF-β-regulated processes from *Drosophila* to humans (Massagué et al., 2005). However, it should be noted that ATF1 and CREB may be only part of the mechanism mediating Smad-dependent repression of these genes in T cells.

ATF family members can act as either transcriptional activators or repressors, depending on their binding partners (Hai and Hartman, 2001). Specifically in T cells, the ATF1 family has been implicated in the repression of various target genes (Penix et al., 1996; Powell et al., 1999). We have shown that Smad2/3

and ATF1 rapidly bind to the promoter region of at least two genes in this program, *GzmB* and *IFNγ*, in response to TGF-β. TGF-β-dependent binding of Smads to a TGF-β-responsive gene promoter and concomitant binding of a Smad-interacting factor to an adjacent site are well-established criteria for direct involvement of the Smad pathway in TGF-β target gene regulation (Massagué et al., 2005).

siRNA-mediated knockdown of ATF1 and CREB in CTLL-2 cells demonstrated a partial loss of TGF-β-mediated repression of GzmB. The residual repression of GzmB by TGF-β suggests either that the knockdown levels attained with these oligonucleotides were not sufficient to completely abrogate repression by TGF-β or that there may be additional inputs by TGF-β on the GzmB promoter, similar to several other well-characterized TGF-β target genes (Massagué et al., 2005). Further analysis is required to determine whether alternative Smad cofactors are involved in repression of GzmB. However, RNAi-mediated depletion of Smad2 and Smad3 causes a block in TGF-β-mediated gene repression, indicating that repression of GzmB by TGF-β is entirely a Smad-dependent effect. While further analysis is required to fully characterize *GzmB* and *IFNγ* as TGF-β-responsive genes, it is interesting that TGF-β appears to coordinate the repression of at least these two cytotoxic genes via ATF1-Smad or CREB-Smad complexes in T lymphocytes.

TGF-β represses the cytotoxic programs, independent of CD8+ T cell activation or growth inhibition

TGF-β can inhibit the expression and production of IL-2 by T cells (McKarns et al., 2004). This effect is confirmed by our transcriptomic profiling showing repression of *IL-2* by TGF-β in primary mouse T cells ex vivo, as well as our observation that TGF-β inhibits IL-2 production and IL-2-mediated T cell proliferation in these cells. However, by exposing these cells to exogenous IL-2 and thus bypassing the block of cell proliferation, we have been able to clearly separate the antimitogenic effect of TGF-β from its effects on the cytotoxic program. In addition, TGF-β can rapidly (within 4 hr) downregulate *GzmB* and *perforin* expression in the IL-2-dependent T cell line CTLL-2, which precedes growth inhibition by 20 hr, further highlighting the independence of the two TGF-β-regulated pathways. Furthermore, our analysis indicates that TGF-β does not alter the overall activation profile of CD8+ T cells, as defined by the cell surface markers CD69, CD44, and CD62L. Consistent with these results, we observed no defect in CD8+ T cell activation upon challenge with TGF-β-producing tumors in vivo. These observations suggest that the CD8+ T cells in vivo undergo proper development in the presence of tumor-derived TGF-β but fail to activate cytotoxic gene expression.

However, as analyzed with the OVA antigen-MHC class I tetramer system, the proportion of tumor-specific T cells was increased when we neutralized tumor-associated TGF-β with a TGF-β trap in our mouse model. This suggests that, similar to in vitro observations, TGF-β regulates both the clonal expansion of CD8+ T cells and CD8+ T cell cytotoxicity in vivo. Inhibition of T cell proliferation and repression of the cytotoxic gene program in T cells are therefore two distinct effects, which, together, would ultimately favor tumor progression.

TGF-β blockade restores cytotoxic functions in tumor-specific T cells

The ability to identify antigen-specific T cells using tetramers of recombinant MHC class I bound to the antigenic peptide has enormously facilitated the analysis of T cell biology under tumor and pathogen challenges (Appay and Rowland-Jones, 2002; Xu and Screaton, 2002). We have combined tetramer technology with T cell profiling to identify the molecular signature regulated by TGF-β, specifically in antigen-reactive T cells in vivo. Our experiments using the EL4-OVA-soluble TβR-II system demonstrate that systemic neutralization of TGF-β protects the tumor-specific activity of CTLs. The ability to recognize and clear EL4-OVA tumor cells in this model system is linked to increased expression of perforin, GzmA, GzmB, and IFNγ but not FasL. The Fas-FasL pathway regulates the primary immune response through activation-induced cell death (AICD). TGF-β1$^{-/-}$ T cells have increased surface levels of FasL, suggesting that regulation of this pathway by TGF-β may be critical for maintaining T cell homeostasis (Wahl et al., 2000). Since neutralization of TGF-β in vivo does not alter FasL expression on antigen-specific CTLs, we conclude that Fas-FasL pathway does not play an essential role in this in vivo tumor clearance model.

The role of perforin and IFNγ in tumor clearance has been demonstrated using perforin- and IFNγR1-deficient mice (Street et al., 2001). However, the essential roles of GzmA and GzmB in tumor clearance in vivo have not been consistently documented (Pardo et al., 2002b; Trapani and Sutton, 2003). Studies have indicated that the nature and quality of the target cell may dictate the requirement for each respective granzyme (Pardo et al., 2002a). In addition, it is becoming increasingly evident that the background strain of the mice plays a critical role in determining which granzymes mediate tumor clearance in vivo (Pardo et al., 2002b). While perforin-deficient mice are highly susceptible to EL4 tumor challenge, GzmAxGzmB doubly deficient mice have not been described. Although the individual effector contributions are not detailed, neutralization of TGF-β activity in vivo leads to restoration of a cytotoxic gene program, which, collectively, would contribute to EL4 tumor clearance.

We also show that T cells that have suffered inhibition by TGF-β can have their proliferation and cytolytic functions restored upon removal of TGF-β and stimulation with IL-2. In contrast, IL-15, a cytokine in the same γc family as IL-2 involved in regulation of CD8+ T cell development and effector function (Schluns and Leofrancois, 2003), could partially rescue proliferation but could not restore cytotoxic gene expression in T cells pretreated with TGF-β. These results suggest that, following TGF-β treatment, IL-2 can rescue overall CD8+ T cell growth and effector functions, while IL-15 rescues only CD8+ T cell expansion. Thus, the immunosuppressive effects of TGF-β are reversible, and treatment with IL-2 is capable of restoring CTL effector function in vitro. Neutralization of TGF-β in vivo may not only allow new tumor-specific T cells to develop, but these cells may also produce enough IL-2 to rescue CTL effector functions.

The ultimate goal of cancer immunotherapy is to restore or enhance the ability of tumor antigen-specific T cells to recognize and eliminate tumor cells in vivo (Blattman and Greenberg, 2004). The paradoxical effects of TGF-β on the tumor cell itself are already well documented, but only recently have the effects of TGF-β on the host immune response been appreciated (Wojtowicz-Praga, 2003). Therapies that target the TGF-β signaling pathway in tumor cells, such as receptor kinase inhibitors and TGF-β-neutralizing proteins, are currently in various stages of clinical development (reviewed in (Dumont and Arteaga [2003];

Yingling et al. [2004]). Our studies suggest that neutralization of the effects of TGF-β on T cells in vivo can restore critical cytotoxic gene responses involved in tumor clearance. A better understanding of the effects of TGF-β on the immune system seems warranted so that new therapies will more comprehensively address the role of TGF-β in cancer development.

Experimental procedures

T cell activation assays

CD4+/CD8+ or CD8+ T cells were purified from mouse spleens using *MACS* magnetic bead cell sorting (Miltenyi Biotec) and activated with plate bound 0.25 μg/ml antiCD3ε plus soluble antiCD28 in the presence or absence of 100 pM TGF-β1 (R & D Systems) typically for 24 hr. Growth inhibition assays were performed as previously described (Chen et al., 2001). RNA was extracted from T cells and Northern analysis was performed as previously described (Chen et al., 2002). The GzmA, GzmB, and perforin cDNAs used as northern probes as well as antiGzmA and antiGzmB rabbit polyclonal serum were gifts from Dr. T.J. Ley (WUSM). ELISA analysis (R & D Systems) was performed in triplicate according to the manufacturer's protocol.

Flow cytometry and tetramer analysis

For flow cytometry assays of in vitro-activated T cells, 1×10^6 cells were stained with the following antibodies: antiCD3 (CALTAG Laboratories), antiCD4, antiCD69, antiCD8α, and antiCD25 (BD Pharmingen). Background levels were determined with isotype-matched control antibodies. Recombinant H-2K^b tetramers bound to OVA-peptide SIINFEKL were produced by the Microchemistry Facility and Tetramer Core Facility at MSKCC. Freshly purified spleen cells were stained with H-2K^b tetramers for 30 min on ice and surface stained with CD8, CD44, CD62L, CD69, and/or FasL antibodies. Cells were then fixed and permeabilized with Cytofix/Cytoperm kit (BD Pharmingen), followed by intracellular staining with GzmB, GzmA (BD Pharmingen), IFNγ (R & D Systems), or perforin (Kamiya Biochemicals) antibodies. A minimum of 100,000 live events were collected in duplicate and analyzed with FloJo6.1 software. Each experiment was performed three times, each with two mice.

Microarray expression analysis

RNA was harvested from primary CD90+ T cells activated for 24 hr as described above, and each sample, in duplicate, was hybridized to an Affymetrix Mouse Genome U74Av2 Microarray (MSKCC Genomics Core). Comparative analysis between TGF-β-treated and untreated T cell samples was performed using Microarray Suite 5.0 software. Expression changes of more than 2.0-fold with p = 0.001 were scored as TGF-β target genes.

Cell lines

Mouse P815 mastocytoma cells, EL4 lymphoma cells, EL4-OVA (E.G7), and CTLL-2 T cells were all purchased from ATCC and cultured accordingly. CTLL-2 cells were supplemented with 0.5 ng/ml rIL-2 every 2 days.

Transcriptional assays and knockdown experiments

CTLL-2 cells were pretreated with 10 μg/ml CHX for 1 hr, prior to treatment with TGF-β for 4 hr. Mouse GzmB promoter constructs (–3000 and –662) were obtained from Dr. M. Cross (University of Leipzig, Germany) or Dr. T.J. Ley (–960/+74;). The –885/+74 and –794/+74 deletion mutants were generated using internal primers and cloned into the pGL2 basic promoter.

Mutation of individual transcription factor sites within the –885/+74 GzmB promoter was performed using the following primers, with the replaced nucleotides underlined: MT ATF1, 5′-GCCAGCCAGCCAGTTTAAGCTTATC ACCAAGT TAATC-3′; MT Oct1/Gata1, 5′-GCCAGTTTAACGTAATCTAGAA GTTAATCGAGA-3′; and MT AML1a, 5′-GAGAGACA CCTCTAGATCTCT GGCCAGC-3′. CTLL-2 cells were transfected via the Amaxa Nucleofection System Buffer R (Program T-28) or Buffer T (Program K-17).

Mouse siRNA oligonucleotides were obtained from the HTS Core Facility. Three sets of oligonucleotide pairs targeting each gene were tested, and knockdown experiments were performed via nucleofection into CTLL-2 cells. Sense strand of the siRNA oligonucleotide sequences for ATF1, CREB1, Smad2, and Smad3 are as follows, respectively: 5′-GCGUUACU AAGUAUGUA AATT-3′, 5′-CCACCCUCAAGAAGUAAUCTT-3′, 5′-CCCA GUAUUGCAGUACUAUTT-3′, and 5′-UCGCCACCUGACUCCUUGUTT-3′. After 48 hr, cells were treated minus and plus TGF-β for 3 hr, and Northern analysis was performed.

ChIP

Primary mouse T cells were activated as described above, minus and plus 100 pM TGF-β for 15 hr. ChIP assays were performed as previously described (Seoane et al., 2002). The antibodies utilized were antiSmad 2/3, antiATF1 (Santa Cruz), and antiCREB (Cell Signaling). The GzmB primer set spans –930 to –585, and the IFNγ primer set utilized spans –366 to –16, relative to the transcriptional start site.

Cytotoxicity and tumor rejection assays

Cytotoxicity assays against mouse P815 mastocytoma tumor cells were performed as previously described (Heusel et al., 1994). Human TβR-II ectodomain construct was generated by insertion of stop codon after amino acid 168 and cloned into a retroviral vector. The mTGF-β1 RNAi oligonucleotides were cloned into retrovial vector: sense, gatccc GGAGACGGAATACA GGGCTttcaagagaAGCCCTGTATTCCGTCTCC; antisense, agcttttccaaaaa GGAGACGGAATACAGGGCTtctcttgaaAGCCCTGTATTCC TCTCCggg. Expression of soluble TβR-II was determined by goat anti-human TβR-II immunoblot (R & D Systems).

Five C57Bl/6 mice per cohort per experiment were injected intraperitoneally with 2.5×10^6 EL4-pRS, EL4-shTGF-β1, EL4/EL4-OVA, or EL4/EL4-OVA soluble TβR-II cells. Mice were observed for signs of tumor development and sacrificed accordingly.

Supplemental data

Supplemental Data include one table and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/5/369/DC1/.

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