CD28 Costimulation Overcomes Transforming Growth Factor-β–Mediated Repression of Proliferation of Redirected Human CD4$^+$ and CD8$^+$ T Cells in an Antitumor Cell Attack

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Abstract

The T-cell–mediated antitumor immune response is frequently repressed in the tumor environment by an immunologic barrier, the predominant mediators of which are thought to be interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). We explored the effect of these cytokines on the individual T-cell effector functions on antigen engagement during an antitumor cell attack. Isolated CD4$^+$ and CD8$^+$ T cells were antigen-specifically redirected toward carcinoembryonic antigen (CEA)-positive tumor cells by expression of a recombinant T-cell receptor (immunoreceptor), which triggers T-cell activation via CD3ζ on binding to CEA. Immunoreceptor-activated T cells secrete IFN-γ, proliferate, and lyse CEA$^+$ but not CEA$^-$ tumor cells. Whereas IL-10 has no direct effect on immunoreceptor-triggered effector functions, TGF-β represses proliferation of both CD4$^+$ and CD8$^+$ T cells but neither IFN-γ secretion nor specific cytolytic activities. CD28 costimulation, however, overcomes TGF-β–mediated repression in T-cell proliferation. Consequently, T cells redirected by a combined CD28-CD3ζ signaling immunoreceptor are largely resistant to TGF-β–mediated repression. This is reflected *in vivo* by a more pronounced antitumor activity of T cells against TGF-β–secreting tumors when redirected by a costimulatory CD28-CD3ζ than by a CD3ζ signaling immunoreceptor. [Cancer Res 2007;67(5):2265–73]

Introduction

Tumor antigen-specific cytotoxic T cells frequently infiltrate the tumor tissue but, although in some tumors in large numbers, without signs of tumor destruction. Several lines of evidence sustain the concept that a growing tumor is building up an immunosuppressive environment in a direct way by secreting suppressive cytokines that act on immunologic effector cells as well as indirectly by inducing regulatory T cells that locally repress an antitumor T-cell attack (1). The immunologic barriers protecting the tumor, however, are thus far not understood in detail. Interleukin (IL)-10 and transforming growth factor-β (TGF-β) produced by tumor cells themselves and/or by infiltrating regulatory T cells are thought to be the major immunosuppressive cytokines accumulating to high concentrations in the tumor environment (2). IL-10 represses multiple immune responses through individual actions on T and B cells, antigen-presenting cells (APC), and other cell types and skews the immune response from Th1 to Th2 (3). T-cell stimulation *in vitro* in the presence of IL-10 leads to long-term anergy and to the generation of negative regulatory T-cell subsets.

A variety of tumors, including gastrointestinal, lung, and mammary carcinomas, highly express TGF-β, thereby triggering tumor progression by loss of growth-inhibitory control and increase in metastatic potential and repressing a specific antitumor immune attack. Whereas the effects of TGF-β on the tumor cells themselves are well documented, the effects on effector immune cells have only recently been appreciated (4, 5). TGF-β limits the effector functions of a variety of immune cells, including tumor antigen-specific cytotoxic T cells and natural and lymphocyte-activated killer cells (2, 6). TGF-β controls T-cell homeostasis by inhibiting T-cell proliferation and activation as well as by inhibiting presentation of antigens on APCs (7). Various mouse tumor models showed that production or activation of TGF-β is one of the most potent mechanisms of tumor cells to prevent elimination by effector immune cells (8, 9). Inhibition of TGF-β signaling in CD8$^+$ T cells may restore tumor immunity because adoptively transferred CD8$^+$ T cells that are made resistant to TGF-β by expression of a dominant-negative TGF-β receptor transgene eliminate efficiently tumor burden (10, 11). Whereas TGF-β–mediated immune suppression in the mouse system was most recently characterized in detail (4), little is known about TGF-β–mediated targeting of human T-cell effector functions during a tumor antigen-specific immune response.

We therefore asked whether IL-10 and TGF-β act directly and/or cooperatively on the initiation of antigen-triggered effector functions of human T cells. To address this issue in a stringently controlled system, we redirected human CD4$^+$ and CD8$^+$ T cells antigen specifically toward tumor cells by expression of a recombinant immunoreceptor that conveys binding specificity for carcinoembryonic antigen (CEA) and intracellular CD3ζ signaling (12). Because CEA binding is mediated by an antibody-derived single-chain Fv (scFv) domain, T-cell activation is triggered independently of MHC, which allows to monitor both CD4$^+$ and CD8$^+$ T-cell activation on binding to the same antigen. Immunoreceptor-triggered T-cell activation is indicated by induction of proliferation, secretion of IFN-γ, and specific lysis of CEA$^+$, but not of CEA$^-$, tumor cells (12). By means of thoroughly defined, antigen-specific, T-cell targeting, we here show that TGF-β does not directly suppress the T-cell immune response but indirectly by repressing T-cell proliferation. TGF-β–mediated repression of T-cell amplification can be overcome by CD28 costimulation, implying combined CD28-CD3ζ signaling of engineered T cells to break the immunologic barrier of tumor tissues.

Materials and Methods

Cell lines and reagents. 293T cells are human embryonic kidney cells that express the SV40 large T antigen (13). LS174T (ATCC CCL 188) is a

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CEA-expressing colon carcinoma cell line, and Colo320 (ATCC CCL 220.1) is a CEA^- cell line. LS174T-B7 cells were derived from LS174T cells by transfection of a bicistronic expression plasmid encoding B7-1 and B7-2 (14). C15A3 cells (kindly provided by Dr. M. Neumaier, Universität Heidelberg, Heidelberg, Germany) were derived from MC38 fibrosarcoma cells by transfection with a plasmid encoding CEA. CEA^+ C15A3 tumor cells secrete ~2.2 ng/mL TGF-β per 10^6 cells in 48 h. Mv-1-Lu (ATCC CCL-64) is a TGF-β–sensitive cell line and was kindly provided by Dr. Silvio Hemmi (Institute of Molecular Biology, University of Zürich, Zürich, Switzerland). MyLa are $CD30^+$ cutaneous lymphoma cells kindly provided by Dr. Reinhard Dummer (Kantonspital, Zürich, Switzerland). OKT3 (ATCC CRL 8001) is a hybridoma cell line that produces the anti-CD3 monoclonal antibody (mAb) OKT3, 15E8 is a hybridoma line that produces the anti-CD28 mAb 15E8 (a kind gift from R. van Lier, Academic Medical Center, Amsterdam, the Netherlands), and BW2064/36 is an internal image anti-idiotypic antibody directed against the anti-CEA scFv BW431/26 (15). All cell lines were cultured in RPMI 1640 supplemented with 10% (v/v) FCS (Life Technologies, Paisley, United Kingdom). CD1 *nu/nu* mice were purchased from Charles River Laboratories (Sulzfeld, Germany). OKT3, 15E8, and BW2064/36 mAbs were affinity purified from hybridoma supernatants using goat anti-mouse IgG1 antibody (Southern Biotechnology, Birmingham, AL) that were immobilized on *N*-hydroxysuccinimide ester–activated Sepharose as recommended by the manufacturer (Amersham Biosciences, Freiburg, Germany). The phycoerythrin (PE)-conjugated and FITC-conjugated anti-CD3, anti-CD4, and anti-CD8 mAbs were purchased from DAKO (Hamburg, Germany). The goat anti-human IgG antibody and its biotin-, FITC-, and PE-conjugated $F(ab')_2$ fragment derivatives were purchased from Southern Biotechnology. The anti-human IFN-γ mAb NIB42 and the biotinylated anti-human IFN-γ mAb 4S.B3 were purchased from BD Biosciences (San Diego, CA). Purified CEA was purchased from Calbiotech (Spring Valley, CA), and bovine submaxillary mucin was from Sigma (Deisenhofen, Germany). Recombinant human IL-2 (Proleukin) was purchased from Chiron GmbH (Ratingen, Germany), recombinant human IL-10 was from ImmunoTools (Friesoythe, Germany), and recombinant TGF-β was from Cell Concepts (Umkirch, Germany). TGF-β bioactivity was monitored by specific lysis of TGF-β–sensitive Mv-1-Lu (16) and A375 cells (17).

Magnetic-activated cell sorting. Peripheral blood lymphocytes from healthy donors were isolated by density centrifugation, and monocytes were depleted by plastic adherence. Nonadherent lymphocytes were washed with cold PBS containing 0.5% (w/v) bovine serum albumin, 1% (v/v) FCS, and 2 mmol/L EDTA. $CD4^+$ and $CD8^+$ T cells were isolated by magnetic-activated cell sorting using human $CD4^+$ and $CD8^+$ microbeads, respectively (Miltenyi, Bergisch Gladbach, Germany). The number of contaminating cells in the isolated $CD4^+$ and $CD8^+$ T-cell populations was <2%.

Generation of chimeric receptors and transduction of T cells. The generation of the retroviral expression cassettes for the CEA-specific immunoreceptors BW431/26scFv-Fc-CD3ζ (#439), BW431/26scFv-Fc-CD28-CD3ζ (#607), and HRS3scFv-Fc-CD3ζ (#523) has been recently described in detail (18). T cells were retrovirally transduced to express the recombinant receptor as described earlier (19). Briefly, retroviral vector DNA was cotransfected with the helper plasmid DNAs pHIT60 and pCOLT (each at 1 μg DNA/1×10^5 cells; ref. 13) into 293T cells for virus production. Isolated $CD4^+$ and $CD8^+$ T cells from the peripheral blood were activated by addition of IL-2 (1,000 units/mL) and OKT3 mAb (100 ng/mL) for 48 h, washed, resuspended in RPMI 1640 with IL-2 (1,000 units/mL), and cocultivated for 48 h with transfected 293T cells. T cells were harvested and receptor expression was monitored by flow cytometry using a PE-conjugated $F(ab')_2$ anti-human IgG1 antibody (1 μg/mL) and a FITC-conjugated anti-CD3 mAb (UCHT-1, 1:20) and analyzed using a FACSCanto cytofluorometer equipped with the FACSDiva software (Becton Dickinson, Mountain View, CA).

Receptor-mediated activation of grafted T cells. T cells were grafted with the recombinant anti-CEA immunoreceptors, cultivated in RPMI 1640 supplemented with 10% (v/v) FCS, without stimuli for 24 h, washed, and cocultivated with tumor cells (2.5×10^4 per well) for 48 h in 96-well round-bottomed plates. Culture supernatants were analyzed for secretion of IFN-γ by ELISA using matched pairs of antibodies specific for IFN-γ (clones NIB42 and B133.5; BD Biosciences). Briefly, IFN-γ in the supernatant was bound to a solid-phase anti-human IFN-γ mAb (1 μg/mL) and detected by a biotinylated anti-human IFN-γ mAb (0.5 μg/mL). The reaction product was visualized by a peroxidase-streptavidin conjugate (1:10,000) and ABTS (both purchased from Roche Diagnostics, Mannheim, Germany) as substrate. The detection limit of the assay is 15 pg/mL IFN-γ. To monitor the cytolytic activity, $CD4^+$ T cells (10^4 receptor-grafted cells per well) and $CD8^+$ T cells (5×10^3 receptor-grafted cells per well) were cocultivated with tumor cells (2.5×10^4 per well) for 48 h in 96-well round-bottomed plates. T cells have been preincubated with IL-2, IL-10, or TGF-β 12 to 48 h before the addition of target cells.

Specific cytotoxicity of receptor-grafted T cells was monitored by a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT)-based colorimetric assay (20). Briefly, XTT reagent (1 mg/mL; Cell Proliferation Kit II, Roche Diagnostics) was added to the cells and incubated for 30 to 90 min at 37°C. Reduction of XTT to formazan by viable tumor cells was monitored colorimetrically. Maximal reduction of XTT was determined as the mean of six wells containing tumor cells only and the background as the mean of six wells containing RPMI 1640 supplemented with 10% (v/v) FCS. The nonspecific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells in the same number as in the corresponding experimental wells. The number of viable tumor cells was calculated as follows:

$$\text{viability}[\%] = \frac{OD(\text{exp. wells} - \text{corresponding number of effector cells})}{OD(\text{tumor cells without effectors} - \text{medium})} \times 100$$

5-Carboxylfluorescein diacetate succinimidyl ester labeling. T cells (1×10^7/mL) with and without grafted immunoreceptor were labeled with 5-carboxylfluorescein diacetate succinimidyl ester (CFSE; 1.25 μmol/L; Molecular Probes, Göttingen, Germany) in PBS supplemented with 0.1% (v/v) FCS on ice for 5 min. Cells were washed in cold RPMI 1640 supplemented with 10% (v/v) FCS and incubated in cold medium for 10 min at room temperature. Finally, cells were washed thrice in RPMI 1640 supplemented with 10% (v/v) FCS, incubated for 24 h at 37°C, and washed once again. CFSE-labeled $CD4^+$ T cells (1×10^4 per well) and $CD8^+$ T cells (5×10^3 per well) were incubated in microtiter plates precoated with the anti-idiotypic mAb BW2064/36 (15) directed against BW431/26scFv, which is the binding domain of the anti-CEA immunoreceptors BW431/26scFv-Fc-CD3ζ (#439) and BW431/26scFv-Fc-CD28-CD3ζ (#607), or mouse IgG1 as control (coating concentration, 5 μg/mL). Cells were incubated with or without TGF-β (10 ng/mL), IL-10 (10 ng/mL), and IL-2 (1,000 units/mL). After 6 days, cells were recovered and the number of cycling, CFSE-labeled cells was determined by flow cytometry.

Immunofluorescence analyses. To monitor granzyme expression, T cells were fixed and permeabilized using Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) and stained with a PE-conjugated anti-granzyme A antibody, a mouse PE-conjugated IgG1 isotype antibody (BD PharMingen, San Diego, CA), or a PE-conjugated anti-granzyme B antibody (Serotec, Oxford, United Kingdom). Cells were analyzed by flow cytometry, and the mean fluorescence intensity (MFI) was determined.

Treatment of CD1 *nu/nu* mice. To test for *in vivo* activity of redirected T cells, CEA^+ C15A3 tumor cells that secrete TGF-β were coinjected (5×10^5 per mouse) together with T cells without or equipped either with the CD3ζ chain receptor #439 or alternatively with the combined CD28-CD3ζ signaling receptor #607 (5×10^5 T cells per mouse) into CD1 *nu/nu* mice (six mice per group).

Results

We monitored the effect of TGF-β and IL-10 on the efficiency of specific activation of human $CD4^+$ and $CD8^+$ T cells. Therefore, $CD4^+$ and $CD8^+$ T cells were isolated from the peripheral blood of healthy donors and grafted with specificity for CEA by expression of the recombinant immunoreceptor BW431/26scFv-Fc-CD3ζ (#439) or alternatively the BW431/26scFv-Fc-CD28-CD3ζ (#607). The

immunoreceptors harbor extracellularly the BW431/26 scFv domain for MHC-independent binding to CEA and intracellularly the CD3ζ or the combined CD28-CD3ζ signaling domain for T-cell activation. Both $CD4^+$ and $CD8^+$ human T cells expressed the recombinant immunoreceptors with high efficiency (Fig. 1). Receptor-grafted T cells were specifically activated by coincubation with CEA^+ LS174T tumor cells in a dose-dependent fashion indicated by an increase in IFN-γ secretion and decrease in viability of CEA^+ LS174T cells showing the cytolytic activity of redirected T cells (Fig. 2*A*). T-cell activation is mediated via the recombinant immunoreceptor because T cells grafted with the anti-CEA immunoreceptor were not activated in the presence of CEA^- Colo320 cells and T cells without immunoreceptor were activated in the presence of neither CEA^+ LS174T nor CEA^- Colo320 cells. For comparison, T cells were grafted with the anti-CD30 immunoreceptor HRS3scFv-Fc-CD3ζ (#523), which harbors the anti-CD30 scFv domain instead of the anti-CEA scFv domain BW431/26. T cells with the anti-CD30 immunoreceptor #523 are activated toward $CD30^+$ MyLa cells but not against CEA^+ LS174T cells, whereas T cells with anti-CEA immunoreceptor #439 are activated against CEA^+ LS174T cells but not against $CD30^+$ MyLa cells (Fig. 2*B*).

Using this assay system, we explored whether added cytokines IL-10, TGF-β, and, for comparison, IL-2 modulate the efficiency of antigen-triggered T-cell effector functions. Addition of IL-10 substantially increased receptor-triggered IFN-γ secretion by $CD8^+$ T cells and, to lower amounts, by $CD4^+$ T cells in the presence of CEA^+ LS174T cells. TGF-β, noteworthy, did not alter the amount of secreted IFN-γ under these stimulatory conditions (Fig. 3*A* and *B*). TGF-β used in these assays is biologically active as it suppressed proliferation of TGF-β–sensitive Mv-1-Lu and A375 cells (data not shown). As controls, T cells without recombinant immunoreceptor and coincubated with LS174T cells did not increase IFN-γ secretion nor did receptor-grafted T cells coincubated with CEA^- Colo320 cells in the presence of neither IL-10 nor TGF-β (data not shown). For comparison, addition of IL-2 furthermore increased in a dose-dependent manner IFN-γ secretion by receptor-grafted $CD4^+$ and $CD8^+$ T cells when coincubated with CEA^+ LS174T cells. The effect is restricted to immunoreceptor-mediated T-cell activation and not due to an unspecific T-cell activation because (*a*) addition of IL-2 to T cells without immunoreceptor and (*b*) coincubation with CEA^- Colo320 in the presence of IL-2 did not induce IFN-γ secretion (Fig. 3*C*).

We now asked whether added cytokines act on receptor-triggered cytolytic activities of T cells. Therefore, $CD8^+$ T cells were grafted with the BW431/26scFv-Fc-CD3ζ immunoreceptor and coincubated with CEA^+ LS174T in the presence of IL-2, IL-10, and TGF-β. As summarized in Fig. 3*D*, the CEA-specific cytolytic activity of receptor-grafted $CD8^+$ T cells toward CEA^+ LS174T cells was not significantly altered in the presence of TGF-β. IL-10 slightly increased the antigen-specific cytolytic activity of $CD8^+$ T cells, whereas IL-2 unspecifically increased the cytolytic activity of both receptor-grafted and nongrafted T cells.

To explore whether B7-CD28 costimulation has an effect on the TGF-β effect on redirected T cells, we used LS174T-B7 cells as target cells that express both B7-1 and B7-2 on the cell surface on transfection (14). Again, TGF-β did not inhibit the cytolytic activity of receptor-grafted T cells (Fig. 3*E*). Consequently, T cells redirected by the immunoreceptor BW431/26scFv-Fc-CD28-CD3ζ (#607) that harbors the costimulatory CD28 domain together with the CD3ζ domain are not inhibited in their lytic activity in the presence of TGF-β. Lysis is specific as CEA^- Colo320 cells were not lysed by T cells with an anti-CEA immunoreceptor, and T cells grafted with a receptor of irrelevant binding specificity [i.e., the anti-CD30 receptor HRS3scFv-Fc-CD3ζ (#523)] did not lyse CEA^+ tumor cells (Fig. 3*E*).

Using cross-titrations, we explored whether IL-10 and TGF-β act in concert on IFN-γ secretion and/or cytolytic activity of redirected T cells. Neither IFN-γ secretion nor specific cytolytic activity of $CD4^+$ and $CD8^+$ T cells was substantially altered on receptor triggering in the presence of both IL-10 and TGF-β (data not shown).

To monitor the effect of added cytokines on receptor-triggered proliferation, $CD4^+$ and $CD8^+$ T cells with and without BW431/26scFv-Fc-CD3ζ immunoreceptor were incubated with the immobilized anti-idiotypic mAb BW2064/36, which is directed against the CEA-binding domain BW431/26scFv of the immunoreceptor. An immobilized, isotype-matched mAb served as control. Receptor-grafted $CD8^+$ as well as $CD4^+$ T cells increased proliferation on

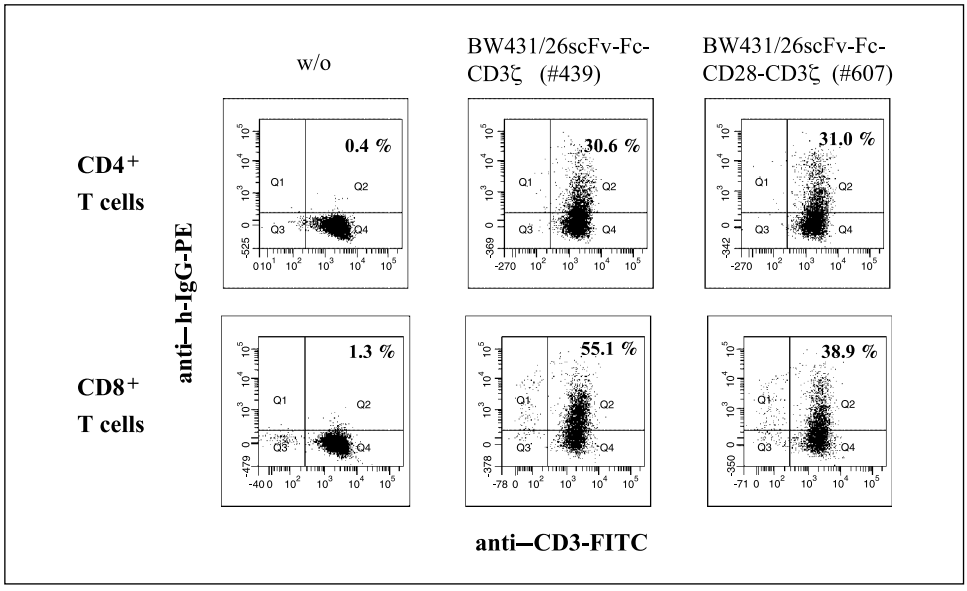

Figure 1. $CD4^+$ and $CD8^+$ T cells are grafted with recombinant anti-CEA immunoreceptors. $CD4^+$ and $CD8^+$ T cells were isolated from peripheral blood by magnetic cell sorting procedures and grafted by retroviral gene transfer with the CEA-specific immunoreceptor BW431/26scFv-Fc-CD3ζ (#439) and BW431/26scFv-Fc-CD28-CD3ζ (#607), respectively, as described in Materials and Methods. To monitor receptor expression, T cells were simultaneously incubated with a PE-conjugated anti-human IgG1 mAb that binds to the extracellular spacer region of the immunoreceptor and with a FITC-conjugated anti-CD3 mAb and analyzed by flow cytometry.

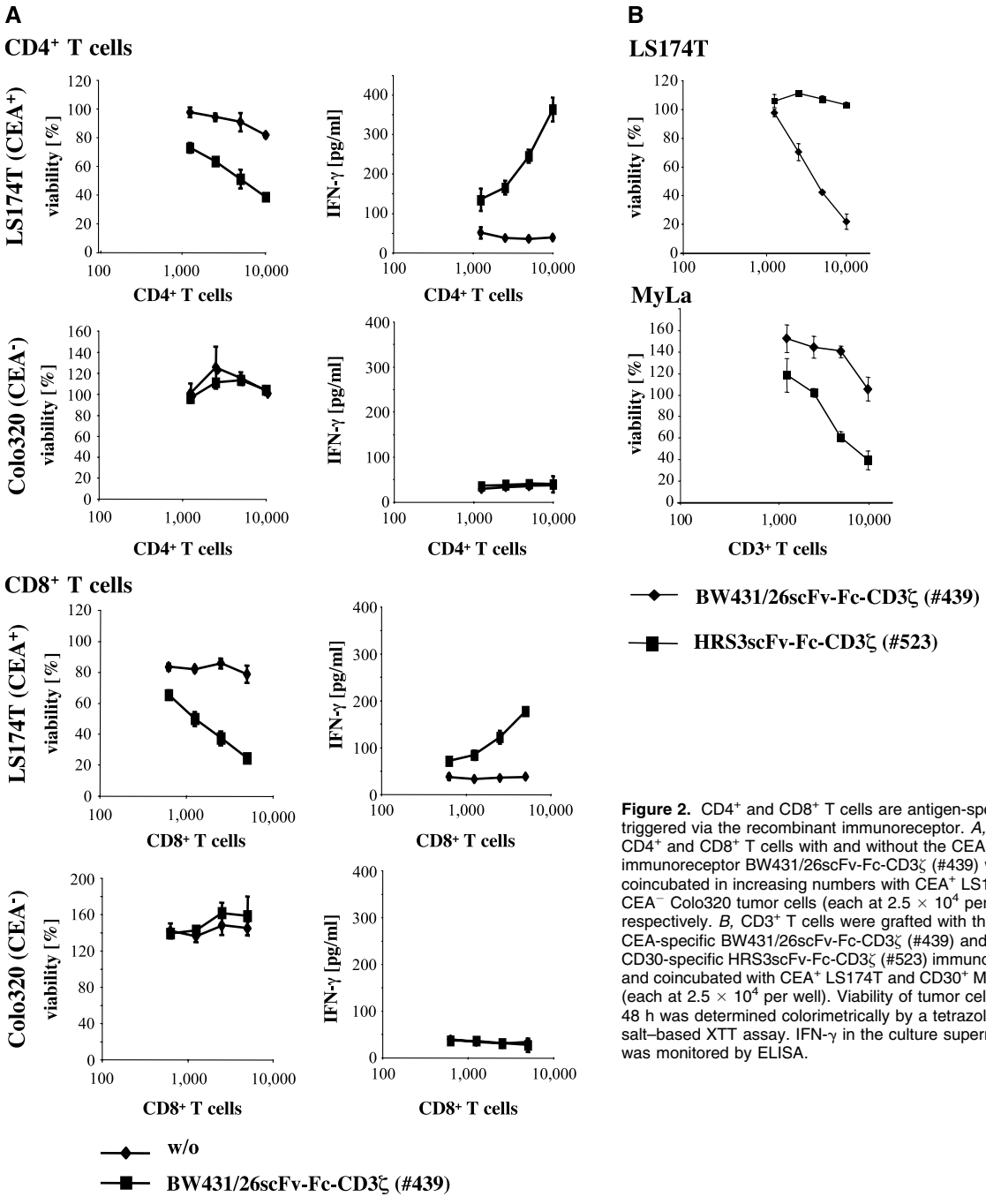

Figure 2. CD4$^+$ and CD8$^+$ T cells are antigen-specifically triggered via the recombinant immunoreceptor. *A,* isolated CD4$^+$ and CD8$^+$ T cells with and without the CEA-specific immunoreceptor BW431/26scFv-Fc-CD3ζ (#439) were coincubated in increasing numbers with CEA$^+$ LS174T and CEA$^-$ Colo320 tumor cells (each at 2.5 × 10^4 per well), respectively. *B,* CD3$^+$ T cells were grafted with the CEA-specific BW431/26scFv-Fc-CD3ζ (#439) and the CD30-specific HRS3scFv-Fc-CD3ζ (#523) immunoreceptor and coincubated with CEA$^+$ LS174T and CD30$^+$ MyLa cells (each at 2.5 × 10^4 per well). Viability of tumor cells after 48 h was determined colorimetrically by a tetrazolium salt–based XTT assay. IFN-γ in the culture supernatant was monitored by ELISA.

specific triggering in contrast to T cells without immunoreceptor (Fig. 4*A*). Addition of TGF-β repressed immunoreceptor-triggered proliferation of both CD4$^+$ and CD8$^+$ T cells, whereas IL-10 did not alter receptor-triggered proliferation. As expected, addition of IL-2 furthermore increased proliferation of immunoreceptor-grafted T cells independently of immunoreceptor triggering. Activation by the anti-idiotypic mAb BW2064/36 is specific for immunoreceptors with the BW431/26 scFv domain because T cells grafted with an irrelevant receptor are not stimulated by mAb BW2064/36 (data not shown).

To explore whether CD28 costimulation can counteract TGF-β–mediated repression in proliferation, we incubated BW431/26scFv-Fc-CD3ζ (#439) receptor-grafted T cells on plates coated with the anti-idiotypic mAb BW2064/36 as antigen together with the agonistic anti-CD28 antibody 15E8. As summarized in Fig. 4*B*, CD28 costimulation partly overcomes TGF-β–mediated repression in proliferation of receptor-grafted T cells on antigen encounter. We therefore asked whether triggering by an immunoreceptor that harbors the costimulatory CD28 in addition to the CD3ζ signaling domain makes T-cell proliferation less sensitive to the repressive

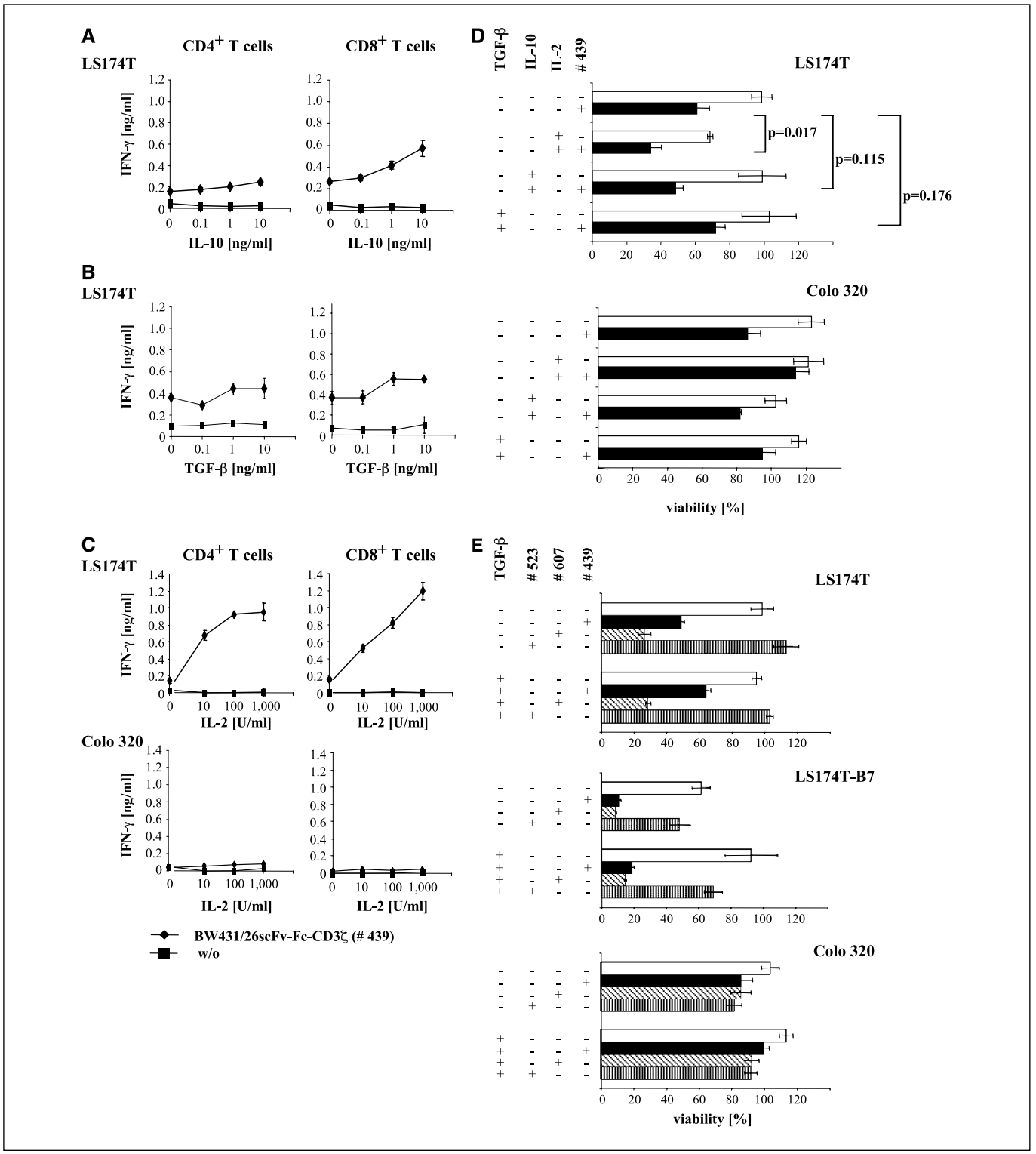

Figure 3. IL-10 and TGF-β act differentially on effector functions of $CD4^+$ and $CD8^+$ T cells redirected by a recombinant immunoreceptor. Isolated $CD4^+$ and $CD8^+$ T cells were retrovirally transduced to express the CEA-specific immunoreceptor BW431/26scFv-Fc-CD3ζ (#439). Receptor-grafted as well as nonmodified T cells (5×10^3 receptor-grafted $CD8^+$ T cells per well; 10^4 receptor-grafted $CD4^+$ T cells per well) were coincubated for 48 h with CEA^+ LS174T or CEA^- Colo320 cells (each at 2.5×10^4 per well), respectively, in the presence of increasing amounts of IL-10 (*A*), TGF-β (*B*), and IL-2 (*C*). IFN-γ in the culture supernatant was monitored by ELISA. *D,* isolated $CD8^+$ T cells were grafted by retroviral gene transfer with the CEA-specific immunoreceptor #439 and coincubated (5×10^3 receptor-expressing T cells per well) with CEA^+ LS174T or CEA^- Colo320 cells (each at 2.5×10^4 per well), respectively, in the presence of IL-2 (1,000 units/mL), IL-10 (10 ng/mL), or TGF-β (10 ng/mL) for 48 h. Essentially the same results were obtained after 4 d of coincubation. *E,* T cells were grafted with the CEA-specific immunoreceptors BW431/26scFv-Fc-CD3ζ (#439) and BW431/26scFv-Fc-CD28-CD3ζ (#607) and with the CD30-specific immunoreceptor HRS3scFv-Fc-CD3ζ (#523). Cells were coincubated (10^4 receptor-expressing T cells per well) with CEA^+ LS174T and CEA^+ LS174T-B7 cells that express B7-1 and B7-2 after transfection and CEA^- Colo320 cells (each at 2.5×10^4 per well), respectively, in the presence or absence of TGF-β (10 ng/mL) for 48 h. Viability of the tumor cells was monitored by an XTT-based assay as described in Materials and Methods. *Points,* mean; *bars,* SE. *P* values are calculated based on a Student's *t* test.

effect of TGF-β. CD4$^+$ and CD8$^+$ T cells were grafted either with the CD3ζ signaling immunoreceptor #439 or alternatively with the costimulatory CD28-CD3ζ immunoreceptor #607 and stimulated via immunoreceptor by incubation with the immobilized anti-idiotypic mAb BW2064/36. In the presence of TGF-β, proliferation of T cells equipped with the costimulatory CD28-CD3ζ receptor #607 was far less repressed than of T cells equipped with the CD3ζ signaling #439 receptor (Fig. 4*C*). Taken together, the data clearly indicate that TGF-β–mediated repression in proliferation of redirected T cells can be overcome by combined CD28-CD3ζ signaling as provided by the immunoreceptor #607.

We monitored the expression of the cytolytic effector molecules granzyme A and B because they represent the major effector molecules by which redirected T cells lyse their target cells (21–23). T cells grafted with the combined CD28-CD3ζ signaling receptor #607 harbor increased levels of both granzyme A and B compared with T cells grafted with the CD3ζ signaling receptor #439 (Fig. 5). Incubation of CD8$^+$ T cells in the presence of TGF-β did not alter substantially the levels of granzyme B expression. This is shown for T cells with the CD3ζ and CD28-CD3ζ signaling immunoreceptor. TGF-β decreases the level of granzyme A in T cells with #607 receptor, however, to levels that are as high as in T cells with or without the #439 receptor. For comparison, IL-2 increased granzyme B expression in CD4$^+$ and CD8$^+$ T cells with and without immunoreceptor. Taken together, the expression levels of granzyme B, which is predominantly involved in mediating cytolytic activities of redirected T cells, are not substantially altered in the presence of TGF-β, reflecting their retained cytolytic activity.

We asked whether T cells grafted with the CD28-CD3ζ costimulatory immunoreceptor are more potent toward a TGF-β–secreting tumor than T cells with a CD3ζ signaling immunoreceptor. Therefore, we coinjected CEA$^+$, TGF-β–secreting C15A3 tumor cells together with T cells equipped either with the CD3ζ chain receptor #439 or alternatively with the combined CD28-CD3ζ signaling

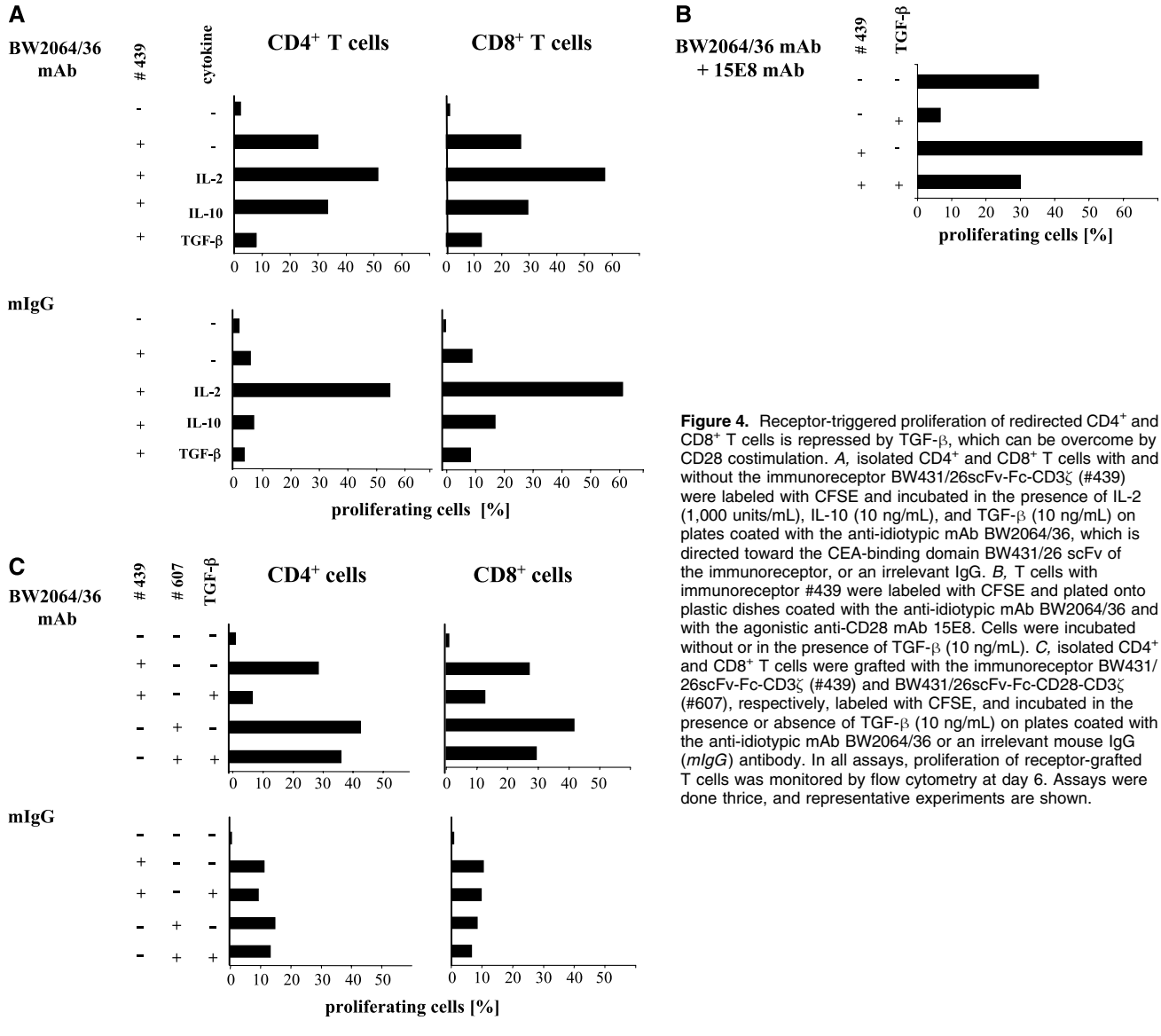

Figure 4. Receptor-triggered proliferation of redirected CD4$^+$ and CD8$^+$ T cells is repressed by TGF-β, which can be overcome by CD28 costimulation. *A,* isolated CD4$^+$ and CD8$^+$ T cells with and without the immunoreceptor BW431/26scFv-Fc-CD3ζ (#439) were labeled with CFSE and incubated in the presence of IL-2 (1,000 units/mL), IL-10 (10 ng/mL), and TGF-β (10 ng/mL) on plates coated with the anti-idiotypic mAb BW2064/36, which is directed toward the CEA-binding domain BW431/26 scFv of the immunoreceptor, or an irrelevant IgG. *B,* T cells with immunoreceptor #439 were labeled with CFSE and plated onto plastic dishes coated with the anti-idiotypic mAb BW2064/36 and with the agonistic anti-CD28 mAb 15E8. Cells were incubated without or in the presence of TGF-β (10 ng/mL). *C,* isolated CD4$^+$ and CD8$^+$ T cells were grafted with the immunoreceptor BW431/26scFv-Fc-CD3ζ (#439) and BW431/26scFv-Fc-CD28-CD3ζ (#607), respectively, labeled with CFSE, and incubated in the presence or absence of TGF-β (10 ng/mL) on plates coated with the anti-idiotypic mAb BW2064/36 or an irrelevant mouse IgG (*mIgG*) antibody. In all assays, proliferation of receptor-grafted T cells was monitored by flow cytometry at day 6. Assays were done thrice, and representative experiments are shown.

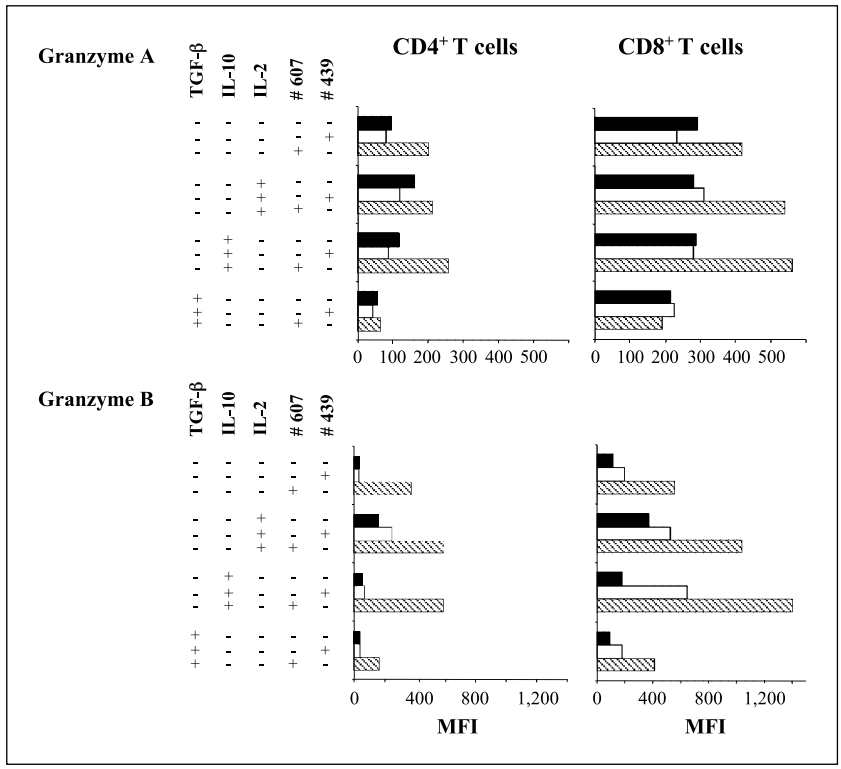

Figure 5. Granzyme A and B in redirected T cells are expressed in altered levels in the presence of added cytokines. Isolated $CD4^+$ and $CD8^+$ T cells were grafted with or without the immunoreceptor BW431/26scFv-Fc-CD28-CD3ζ (#607) and BW431/26scFv-Fc-CD3ζ (#439), respectively, and incubated without added cytokines or in the presence of IL-2 (1,000 units/mL), IL-10 (10 ng/mL), or TGF-β (10 ng/mL) for 2 d. Expression of granzyme A and B in receptor-grafted T cells was monitored by flow cytometry, and the MFI was recorded.

receptor #607 into CD1 *nu/nu* mice. Mice that received T cells with the CD3ζ signaling receptor developed more frequently a tumor compared with mice receiving T cells with the costimulatory CD28-CD3ζ receptor (Fig. 6), indicating a stronger antitumor activity of the costimulatory receptor toward TGF-β–secreting tumor cells than the CD3ζ signaling receptor. As control, T cells without immunoreceptor had minor effects on tumor growth *in vivo*. Taken together, the mouse model sustains our conclusion that T cells redirected by a costimulatory CD28-CD3ζ immunoreceptor are more effective toward a TGF-β–secreting tumor than T cells redirected by a CD3ζ signaling receptor.

Discussion

We here characterized the effect of the immunosuppressive cytokines IL-10 and TGF-β on the execution of effector functions of human engineered $CD4^+$ and $CD8^+$ T cells that are redirected in an antigen-specific fashion toward CEA^+ tumor cells. Because the recombinant immunoreceptor binds antigen via a scFv antibody and thus independently of MHC presentation, $CD4^+$ as well as $CD8^+$ T cells are activated by the same immunoreceptor toward the same antigen. Our main observations are that IL-10, at least in those concentrations tested, increased IFN-γ secretion but did not alter proliferation or specific cytolytic activities of activated $CD4^+$ and $CD8^+$ T cells when stimulated via a strong CD3ζ signaling immunoreceptor. TGF-β, in contrast, did not substantially alter IFN-γ secretion and cytolytic activities but repressed induction of proliferation of redirected T cells. This implies a more specific mechanism by which TGF-β acts on activated T cells than by an overall repression of T-cell effector functions. Whereas numerous studies show that TGF-β is essential to maintain T-cell homeostasis, the mechanisms by which TGF-β regulates T-cell expansion remain poorly understood in detail. Several lines of evidence imply that TGF-β signals through multiple pathways and multiple intracellular effector molecules to inhibit proliferation of activated T cells. One of the intracellular effector molecules, Smad3, was shown to be essential to repress T-cell receptor (TCR)/CD3-induced proliferation (24). TGF-β moreover suppresses the steady-state level of IL-2 on TCR/CD28 signaling in a Smad3-dependent manner. IL-2–dependent T-cell proliferation, however, is repressed by TGF-β through a Smad3-independent pathway. On the other hand, TGF-β cooperates with TCR signaling to increase IL-2 receptor α-chain expression (25). Our data on redirected human T cells are partly in contrast to recent results reported for murine T cells (4). In the murine system, TGF-β directly represses the expression of IFN-γ as well as of perforin, granzyme A and B, and Fas, resulting in suppressed cytotoxicity of $CD8^+$ T cells. The latter is independent of growth inhibition by TGF-β. The difference to our observations on human T cells may be attributed to the fact that we used strongly preactivated T cells in contrast to naive T cells to allow retroviral transduction and immunoreceptor expression.

Our second major observation is that CD28 costimulation reduces the repressive effect of TGF-β on redirected $CD4^+$ as well as $CD8^+$ T cells. The costimulatory signal can be combined with the primary signal into one combined signaling immunoreceptor, providing a strong CD28 signal simultaneously to the CD3ζ signal. Gunnlaugsdottir et al. (26) reported that only T cells receiving strong CD3 stimulation become less responsive toward TGF-β in the presence of CD28 costimulation, indicating that the result of CD28 counteraction depends on the avidity of the TCR/CD3 signal. In that report, naive cord blood cells were used in contrast to activated T cells we used in this study. In the situation of signaling through recombinant immunoreceptors, moreover, T cells bind antigen with high affinity via an antibody-derived binding domain compared with low-avidity interactions of the physiologic TCR/CD3 complex. In this context, our results are in accordance to those reported by

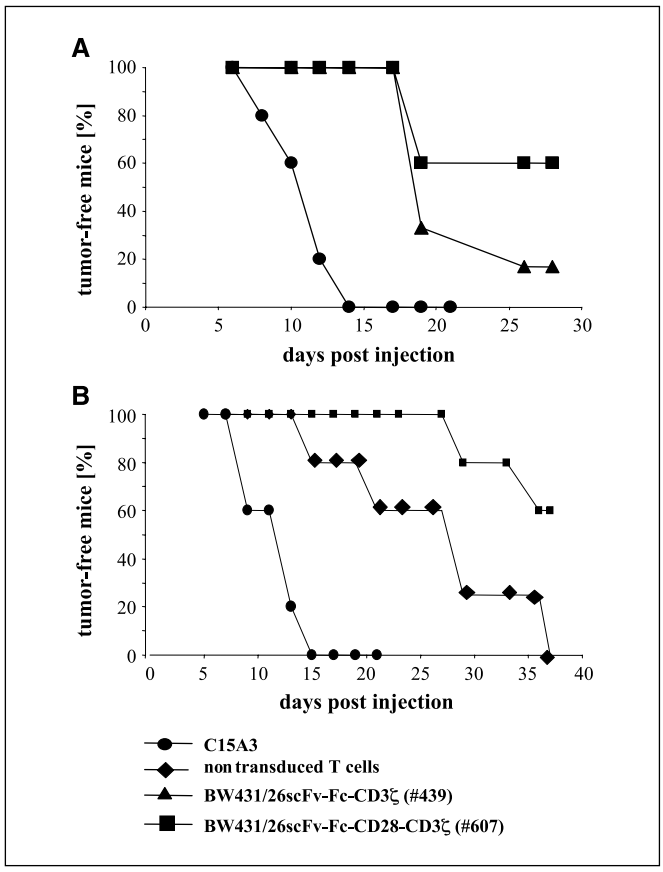

Figure 6. The costimulatory CD28-CD3ζ immunoreceptor #607 mediates a stronger antitumor activity than the CD3ζ signaling receptor #439 toward TGF-β–secreting C15A3 tumor cells *in vivo*. C15A3 tumor cells were s.c. coinjected with or without the same number of $CD3^+$ T cells into CD1 *nu/nu* mice (5×10^5 per mouse; six mice per group) without or equipped either with the BW431/26scFv-Fc-CD3ζ (#439) or alternatively with the BW431/26scFv-Fc-CD28-CD3ζ (#607) immunoreceptor. Tumor growth was monitored every 2 d. Mice with a lesion <100 mm^3 were taken tumor-free.

Tzachanis et al. (27) that Tob, a negative regulator of T-cell activation, is down-regulated during strong stimulatory conditions but not when stimulated suboptimally via TCR/CD3 or CD28 alone. Tob acts as corepressor with the Smad2-Smad4 complex, which is formed on TGF-β signaling, and facilitates binding to the IL-2 promoter, thereby repressing IL-2 transcription. This mechanism, however, does not explain how TGF-β inhibits proliferation and how it is counteracted by strong CD28 signaling. Because Tob shares other inhibitory functions of TGF-β, some of the additional effects may result from a cross-talk between TGF-β and Tob. Moreover, $CD8^+$ T cells express lower amounts of Tob than $CD4^+$ T cells, making them less responsive to CD28 costimulation (27). In our study, however, we found activated $CD4^+$ and $CD8^+$ T cells responding nearly similar to CD28 costimulation. In contrast to activated human T cells analyzed here, in murine naive $CD4^+$ T cells, TGF-β was reported to inhibit proliferation and IL-2 secretion on CD3 stimulation, whereas CD28 costimulation enhanced proliferation while still repressing IL-2 secretion (28). Taken together, there obviously exists a regulatory system where CD28 costimulation can counteract TGF-β–mediated repression of T-cell proliferation. In the physiologic situation, the strength of the CD28 signal may differentiate between weak and nonspecific stimuli versus strong and specific stimuli, resulting in no or successful counteraction of TGF-β–mediated repression, respectively. In the situation of engineered, activated T cells redirected by high-affinity immunoreceptors that provide a strong costimulatory signal, TGF-β–mediated repression in proliferation is successfully counteracted by CD28 signaling.

Results presented here have major implications for the design of redirected T cells that enter the immunosuppressive environment of tumors. Although there seems to be no direct effect on cytolytic activities, T cells activated via a low-avidity TCR/CD3ζ signal alone are likely to be repressed by TGF-β in proliferation. This effect, however, will indirectly result in repression of the antitumor immune response. To overcome the TGF-β–mediated repression in proliferation, we therefore suggest to provide a strong CD28 costimulus simultaneously with the TCR/CD3ζ signal to redirected effector T cells.

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