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Tumor-Specific T Cell Activation by Recombinant Immunoreceptors: CD3ζ Signaling and CD28 Costimulation Are Simultaneously Required for Efficient IL-2 Secretion and Can Be Integrated Into One Combined CD28/CD3ζ Signaling Receptor Molecule[1]

Andreas Hombach,* Anja Wieczarkowiecz,* Thomas Marquardt,* Claudia Heuser,* Loretta Usai,[2]* Christoph Pohl,[†] Barbara Seliger,[‡] and Hinrich Abken[3]*

Recombinant immunoreceptors with specificity for the carcinoembryonic Ag (CEA) can redirect grafted T cells to a MHC/Ag-independent antitumor response. To analyze receptor-mediated cellular activation in the context of CD28 costimulation, we generated: 1) CEA$^+$ colorectal tumor cells that express simultaneously B7-1 and B7-2, and 2) CEA-specific immunoreceptors that harbor intracellularly the signaling moities either of CD28 (BW431/26-scFv-Fc-CD28), CD3ζ (BW431/26-scFv-Fc-CD3ζ), or FcεRIγ (BW431/26-scFv-Fc-γ). By retroviral gene transfer, we grafted activated T cells from the peripheral blood with these immunoreceptors. T cells that express the FcεRIγ or CD3ζ signaling receptor lysed specifically CEA$^+$ tumor cells and secreted high amounts of IFN-γ upon receptor cross-linking, whereas anti-CEA-CD28 receptor-grafted T cells did not, indicating that CD28 signaling alone is not sufficient for efficient T cell activation. CD28 costimulation did not affect cytolysis by T cells equipped with γ- or ζ-signaling receptors, but enhanced both IFN-γ secretion and proliferation. CD28 costimulation, however, was required for efficient IL-2 secretion of anti-CEA-γ receptor-grafted T cells. Both purified CD4$^+$ and CD8$^+$ T cells grafted with immunoreceptors required CD28 costimulation for complete T cell activation. We integrated both CD28 and CD3ζ signaling domains into one combined immunoreceptor molecule (BW431/26-scFv-Fc-CD28/CD3ζ) with dual signaling properties. T cells grafted with the combined CD28/CD3ζ signaling receptor secreted high amounts of IL-2 upon Ag binding without exogenous B7/CD28 costimulation, demonstrating that both MHC-independent cellular activation and CD28 costimulation for complete T cell activation can be delivered by one recombinant receptor molecule. *The Journal of Immunology*, **2001, 167: 6123–6131.**

The immunoreceptor strategy for adoptive immunotherapy is based on grafting T cells with rTCRs that bind Ag by an Ab-derived domain and induce cellular activation by an intracellular signaling domain. The strategy thereby combines the advantages of MHC-independent binding to Ag with efficient T cell activation upon specific binding to the receptor ligand (1–3). The Ag binding domain of the receptor consists of a single-chain Ab fragment (scFv)[4] derived from a mAb; the intracellular signaling domain is derived from the cytoplasmic part of a membrane-bound receptor to induce cellular activation, e.g., the FcεRI receptor γ-chain or the CD3ζ chain. T cells engrafted with the recombinant immunoreceptor induce an Ag-specific, MHC-independent immune response upon Ag-mediated receptor cross-linking (for review, see Refs. 4 and 5).

According to the dual signal model of T cell activation, a costimulatory signal in addition to signaling through the TCR/CD3 complex is required for efficient activation of resting T cells, resulting in cellular proliferation, cytokine secretion, CTL-mediated target cell lysis, and prevention of activation-induced anergy (for review, see Refs. 6 and 7). Resting T cells, however, can be alternatively activated via B7-independent pathways or even without any costimulation (8, 9). Accordingly, analyses of CD28-deficient mice suggest that CD28 preferentially amplifies and sustains a primary T cell response (10) and lowers the amount of Ag required to achieve full cellular activation (11). In contrast to resting T cells, in completely activated T cells Ag-specific cytolysis via the TCR/CD3 complex appears to be independent of CD28/B7 costimulation. The role of CD28 costimulation in preactivated T cells, however, is not yet completely resolved. Particularly, proliferation of CD8$^+$ T cells was demonstrated to be uncoupled from their cytolytic activity, but was substantially enhanced by B7 costimulation (12), indicating that each T cell activation parameter seems to be differentially affected by B7/CD28 costimulation.

From the viewpoint of adoptive immunotherapy, a long lasting antitumor response of completely activated T cells grafted with the Ag-specific immunoreceptor will be crucial for the therapeutic efficacy that requires, in addition to highly efficient target cell lysis,

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[4] Abbreviations used in this paper: scFv, single-chain Ab fragment; CEA, carcinoembryonic Ag; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfonyl)-5[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide.

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proliferation and cytokine secretion of grafted T cells. To address this issue, we explored the role of CD28 costimulation for recombinant immunoreceptor-mediated T cell signaling for use in adoptive immunotherapy. Preactivated T cells were retrovirally grafted with a panel of chimeric receptors that display Ab-like specificity for carcinoembryonic Ag (CEA) and harbor intracellularly the signaling moieties of either 1) CD28 (BW431/26-scFv-Fc-CD28), 2) CD3ζ (BW431/26-scFv-Fc-CD3ζ), 3) FcεRIγ (BW431/26-scFv-Fc-γ), or 4) both CD28 and CD3ζ combined in one receptor molecule (BW431/26-scFv-Fc-CD28/CD3ζ). Utilizing T cells grafted with these recombinant receptors and CEA^+ colorectal tumor cells that express both B7-1 and B7-2, we demonstrate in this study that 1) cellular proliferation and, moreover, Ag-induced IL-2 secretion of grafted T cells require CD28 costimulation, and 2) CD28 costimulation can be delivered together with CD3ζ signaling in a combined immunoreceptor molecule to induce complete cellular activation.

Materials and Methods

Cell lines and Abs

LS174T (ATCC CCL 188) is a CEA-expressing colon carcinoma cell line. The anti-CEA mAb BW431/26, the anti-HRS3 idiotypic mAb 9G10, and the anti-idiotypic mAb BW2064/36 with specificity for the anti-CEA mAb were described elsewhere (13–15). The anti-CD3 mAb OKT3 was obtained from American Type Culture Collection (ATCC, Manassas, VA; ATCC CRL 8001). Hybridoma cells 15E8 that produce an anti-CD28 mAb were kindly provided by R. van Lier (NCB, Amsterdam, The Netherlands). The cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS (all from Sigma, Deisenhofen, Germany). Abs were purified from murine ascites and cell culture supernatants utilizing an agarose-coupled anti-mouse IgG Ab (Sigma). The anti-CEA mAb CEJ065, the FITC-conjugated anti-B7-1 mAb MAB104, and the PE-conjugated anti-B7-2 mAb HA5.2B7 were purchased from Coulter-Immunotech (Hamburg, Germany). The PE-conjugated anti-CD3 mAb UCHT-1, the PE-conjugated anti-CD4 mAb MT310, and the PE- and FITC-conjugated anti-CD8 mAb C8/144B, respectively, were purchased from Dako (Hamburg, Germany). FITC-conjugated $F(ab')_2$ anti-human IgG1 and anti-mouse IgG1 Abs were purchased from Southern Biotechnology (Birmingham, AL). The anti-human IFN-γ mAb NIB42 and the biotinylated anti-human IFN-γ mAb 4S.B3 were purchased from PharMingen (San Diego, CA).

Generation of B7 transfectants

The bicistronic expression plasmid pCB/neo contains the coding sequences for the B7-1 molecule and the B7-2 molecule, linked by an internal ribosomal entry site sequence, for simultaneous expression of both B7-1 and B7-2 under control of the CMV early promoter/enhancer (16). The colorectal carcinoma cell line LS174T was transfected with the pCB/neo DNA utilizing FuGENE transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. After culture for 2 days, transfected cells were selected in the presence of G418 (2 mg/ml; Sigma) and subcloned by limiting dilution techniques. Simultaneous expression of B7-1 and B7-2 on the surface of transfected cells was determined by flow cytometry analysis, as described below.

Generation of chimeric receptors and transduction of peripheral blood T cells

The generation and expression of the CEA-specific BW431/26-scFv-Fc-γ (438) and -CD3ζ (439) receptors in peripheral blood T cells were recently described in detail (17–20). The recombinant BW431/26-scFv-Fc-CD28 (637) and BW431/26-scFv-Fc-CD28/CD3ζ (607) receptors were generated as follows: Briefly, the cDNA coding for the transmembrane and intracellular part of CD28 (aa 135–202) was amplified by PCR utilizing CD28 cDNA as template (21) and flanked with *Bam*HI (5′) and *Xho*I (3′) restriction sites using the primer oligonucleotides 1-CD28-*Bam*HI-sense and 2-CD28-*Xho*I-antisense (Table I). The PCR product was digested and inserted into the Moloney murine leukemia virus-derived retroviral expression vector pBULLET (22) at the *Bam*HI and *Xho*I sites. To generate the cDNA coding for the chimeric CD28/CD3ζ signaling domain, the cDNA of the transmembrane and intracellular part of CD28 and the intracellular part of CD3ζ comprising aa 135–202 and 29–142, respectively, were amplified by PCR utilizing CD28 and anti-CEA-ζ receptor (439) cDNA as templates and oligonucleotides 1-CD28-*Bam*HI-sense, 3-CD28-CD3ζ-antisense, 4-CD28-CD3ζ-sense, and 5-CD3ζ-*Xho*I-antisense as primers (Table I). Herewith, the CD28 and CD3ζ sequences were flanked by overlapping sequences. The rCD28/CD3ζ cDNA sequences were assembled by a PCR reaction, reamplified utilizing the primer oligonucleotides 1-CD28-*Bam*HI-sense and 5-CD3ζ-*Xho*I-antisense introducing *Bam*HI and *Xho*I restriction sites, and inserted into the retroviral expression vector pBULLET, as described above. The sequences coding for the extracellular scFv binding and IgG1 Fc C domains were amplified by PCR utilizing the anti-CEA-ζ receptor (439) cDNA as template and flanked by *Nco*I and *Bgl*II restriction sites by the oligonucleotides 6-Lκ-*Nco*I-sense and 7-hIgG1Fc-*Bgl*II-antisense (Table I). The PCR product was digested with *Nco*I and *Bgl*II and inserted into the *Nco*I and *Bam*HI restriction sites of the retroviral expression vector pBULLET containing the cDNA sequences for the transmembrane and intracellular part of CD28 and CD28/CD3ζ, respectively. The final chimeric receptor cDNAs were designated BW431/26-scFv-Fc-CD28 (637) and BW431/26-scFv-Fc-CD28/CD3ζ (607), respectively. To generate gibbon ape leukemia virus-pseudotyped retrovirus for infection of peripheral blood T cells, the retroviral expression vector DNA (6 μg DNA) was cotransfected with the retroviral helper plasmid DNAs pHIT60 and pCOLT (each 6 μg DNA) into 293T cells by calcium phosphate coprecipitation. pHIT60 DNA encodes the murine leukemia virus *gag* and *pol* genes, whereas pCOLT DNA encodes the gibbon ape leukemia virus-envelope gene under control of the CMV promotor/enhancer (22). Cotransfection results in transient production of high titers of infectious retrovirus. PBLs from healthy donors were isolated by density centrifugation and cultured for 48 h in RPMI 1640 medium supplemented with 10% FCS in the presence of IL-2 (400 U/ml; Endogen, Woburn, MA) and OKT3 mAb (100 ng/ml). Cells were harvested, washed, resuspended in medium with IL-2 (400 U/ml), and cocultured for 48 h with transiently transfected 293T cells. T cells were harvested and receptor expression was monitored by flow cytometric analysis.

Magnetic activated cell sorting (MACS)

$CD4^+$ and $CD8^+$ T cells were isolated from the peripheral blood by MACS utilizing magnetic beads-conjugated anti-CD4 and anti-CD8 mAbs, respectively (both Miltenyi, Bergisch Gladbach, Germany). Briefly, PBLs 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) BSA, 1% (v/v) FCS, and 0.1 M EDTA, and incubated for 15 min on ice with either magnetic beads-conjugated anti-CD4 or anti-CD8 mAbs, according to the manufacturer's recommendations. The cells were washed twice with cold PBS, 0.5% (w/v) BSA, and 0.1 M EDTA, and separated on magnetic columns in a mini-MACS separator (Miltenyi). The number of positively enriched $CD4^+$ and $CD8^+$ T cells was determined by two-color flow cytometry utilizing FITC- and PE-conjugated anti-CD4 and anti-CD8 mAbs. The number of contaminating $CD8^+$ and $CD4^+$ cells was lower than 2% in the population of enriched $CD4^+$ and $CD8^+$ cells, respectively. MACS-enriched T cells were washed, cultured for 48 h in RPMI 1640 medium supplemented with

Table I. *Oligonucleotide primer for the generation of recombinant immunoreceptors*[a]

Name	Sequence
1-CD28-*Bam*HI-sense	5′-CTACTCGAGGATCAGGAGCGATAGGCTGCGAA-3′
2-CD28-*Xho*I-antisense	5′-CTGGATCCCTACGTAATGCTCAGGCTGCTCTTGGCT-3′
3-CD28-CD3ζ-antisense	5′-**GTCTGCGCTCCTGCTGAACTTCACTCTCAGGGAGCGATAGG**CTGCGAAGTC-3′
4-CD28-CD3ζ-sense	5′-**CCTATCGCTCCCTGAGAGTGAAGTTCAGCAGG-AGC**-3′
5-CD3ζ-*Xho*I-antisense	5′-CTACTCGAGGATTAGCGAGGGGGCAGGGC-3′
6-Lκ-*Nco*I-sense	5′-CTACGTACCATGGATTTTCAGGTGCAGATTTTC-3′
7-hIgG1Fc-*Bgl*II-antisense	5′-CCCACCCAGATCTTTTTTACCCAGAGACAGGGAGAG-GCTCTTCTG-3′

[a] Restriction sites are underlined. CD28 and CD3ζ overlapping sequences are in bold face.

10% (v/v) FCS, 400 U/ml IL-2, and 100 ng/ml anti-CD3 mAb OKT3, and grafted with recombinant receptors, as described above.

Immunofluorescence analysis

Receptor-grafted T cells were identified by two-color immunofluorescence utilizing a FITC-conjugated $F(ab')_2$ anti-human IgG1 Ab (2 μg/ml) and a PE-conjugated anti-CD3 mAb (UCHT-1, 1:200). Expression of B7-1 and B7-2 was determined using FITC-conjugated anti-B7-1 (MAB104) and a PE-conjugated anti-B7-2 mAb (HA5.2B7). CEA expression was monitored by incubation with the anti-CEA mAb CEJ065 (10 μg/ml) that was detected by a FITC-conjugated $F(ab')_2$ anti-mouse IgG1 Ab (2 μg/ml). Immunofluorescence was analyzed using a FACScan cytofluorometer equipped with the CellQuest research software (Becton Dickinson, Mountain View, CA).

Stimulation of receptor-grafted peripheral blood T cells

Microtiter plates were coated with several combinations of the anti-CD28 mAb 15E8 (2 μg/ml), the anti-CD3 mAb OKT3 (2 μg/ml), the anti-BW431/26 idiotypic Ab BW2064/36 (4 μg/ml), and an IgG1 control mAb (4 μg/ml) (PharMingen). Transduced or nontransduced peripheral blood T cells (1×10^5 cells/well) were incubated for 48 h at 37°C in coated microtiter plates. Alternatively, receptor-grafted and nontransduced T cells ($0.016–10 \times 10^4$/well) were cocultivated for 48 h with B7-transfected CEA^+ colon carcinoma cells that express both B7-1 and B7-2 (LS174T-B7) and nontransfected CEA^+ colon carcinoma cells (LS174T) (5×10^4/well), respectively. The culture supernatants were analyzed by ELISA for the presence of IFN-γ and IL-2. Briefly, IFN-γ was bound by 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). IL-2 was bound by a solid-phase anti-human IL-2 Ab (1:250) and detected by a biotinylated anti-human IL-2 Ab (1:250) (OptEIA-Set; PharMingen). The reaction product was visualized by a peroxidase-streptavidin conjugate (1:10,000) and ABTS (both purchased from Roche Diagnostics) as a substrate.

Cell proliferation of PKH26-labeled cells

The membrane of receptor-grafted and nontransduced blood lymphocytes was labeled with the red fluorescent dye PKH26 (Sigma), as recently described (23, 24). PKH26-labeled, receptor-grafted, and nontransduced lymphocytes, respectively, were cocultured for 72 h with B7 transfected and nontransfected CEA^+ colon carcinoma cells (5×10^4 cells/well), respectively. Nonadherent PBLs were harvested, stained with a FITC-conjugated anti-human IgG1 Ab, and analyzed by two-color flow cytometry. The lymphocyte population was defined by setting forward and side scatter parameters; receptor-grafted T cells were defined by green fluorescence. Dead cells were excluded from analysis by staining with propidium iodide. Cell division results in reduced intensity of the membrane dye PKH26. Proliferating cells were monitored by PKH26 fluorescence intensity, and histogram markers were set with >97.5% of freshly labeled viable lymphocytes laying outside the defined histogram region.

2,3-Bis(2-methoxy-4-nitro-5-sulfonyl)-5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (XTT)-based cytotoxicity assay

Specific cytotoxicity of receptor-grafted T cells to target cells was monitored by a XTT-based colorimetric assay, according to the method of Jost et al. (25). Briefly, receptor-grafted and nontransduced T cells were cocultivated in round-bottom microtiter plates with B7-transfected (LS17T-B7) and nontransfected (LS174T) CEA^+ tumor cells, respectively, as described above. After 48 h, cells were incubated with XTT reagent (1 mg/ml; Cell Proliferation Kit II; Roche Diagnostics) for 90 min at 37°C. Reduction of XTT to formazan by viable tumor cells was monitored colorimetrically at an adsorbance wavelength of 450 nm and a reference wavelength of 630 nm. Maximal reduction of XTT was determined as the mean of six wells containing tumor cells only, the background as the mean of six wells containing RPMI 1640, 10% FCS. The nonspecific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells alone, in the same number as in the corresponding experimental wells. The viability of tumor cells (%) was calculated as follows:

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

To test for specificity of receptor-mediated lysis of CEA^+ tumor cells, the assay was additionally performed in the presence of the anti-BW431/26 idiotypic mAb BW2064/36 and in the presence of the anti-HRS3 idiotypic mAb 9G10 (each 2 μg/ml) as a control. All assays were done in triplicate.

Results

Expression of recombinant immunoreceptors in PBLs

We generated a panel of recombinant immunoreceptors that harbor extracellularly the same Ag binding domain with specificity for CEA, but intracellularly different transmembrane and signaling domains derived from the CD3ζ chain, the FcϵRIγ chain, and the CD28 coreceptor, respectively (Fig. 1). To combine the CD28 and CD3ζ activation motifs in one receptor molecule, we fused the DNA sequences for the transmembrane and intracellular part of CD28 to the intracellular part of the CD3ζ chain. Peripheral blood T cells were preactivated and retrovirally grafted with the recombinant receptors, as described in *Materials and Methods*. Transduced T cells that express the anti-CEA receptor were identified by two-color fluorescence utilizing anti-CD3 mAb and anti-human IgG Fc Ab that detects the extracellular CH2CH3 C domain of the receptor. As demonstrated in Fig. 2, the recombinant receptors were expressed in peripheral blood T cells with nearly similar efficiency.

Specific activation of anti-CEA-Fc-γR-grafted T cells upon coculture with B7-positive tumor cells

We asked whether B7 expression on target cells modulates 1) cytokine secretion of receptor-grafted T cells, 2) specific cytolysis of target cells, and 3) Ag-driven T cell proliferation upon receptor cross-linking. We transfected the CEA^+ colorectal cancer line LS174T with pCB7neo DNA that contains the expression cassette for both B7-1 and B7-2. Transfected cells, designated LS174T-B7, simultaneously express B7-1 and B7-2, as demonstrated by flow cytometry (Fig. 3). To monitor Ag-driven cellular activation, we cocultured nontransduced T cells and T cells grafted with the BW431/26-scFv-Fc-γ (438) receptor together with B7-expressing and nontransfected LS174T cells and recorded the cytolytic activity and the amount of IFN-γ and IL-2 secretion of grafted T cells. Coincubation of receptor-grafted T cells with CEA^+ LS174T tumor cells resulted in highly efficient lysis of CEA^+ tumor cells, whereas T cells lacking the CEA-specific receptor were poorly cytolytic (Fig. 4*A*). Coincubation with LS174T-B7 tumor cells that express B7-1 and B7-2 did not alter the cytolytic efficacy of receptor-grafted T cells. IFN-γ secretion, however, is dramatically increased after coculture of receptor-grafted T cells with $B7^+$ tumor cells compared with coincubation with $B7^-$ tumor cells (Fig. 4*A*). Notably, receptor-grafted T cells secrete high amounts of IL-2 upon coincubation with $B7^+$ LS174T-B7 cells,

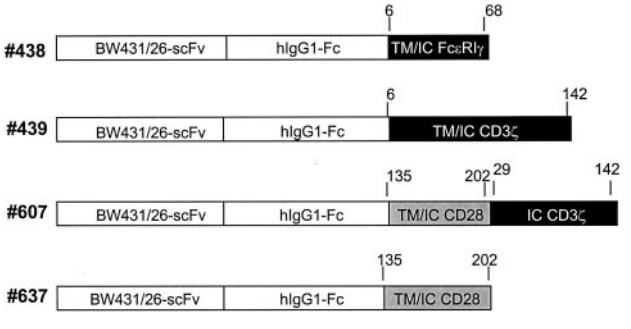

FIGURE 1. Expression cassettes for the recombinant anti-CEA immunoreceptors used in this study. The numbers indicate the amino acids constituting the transmembrane (TM) and intracellular (IC) signaling domains of the immunoreceptor.

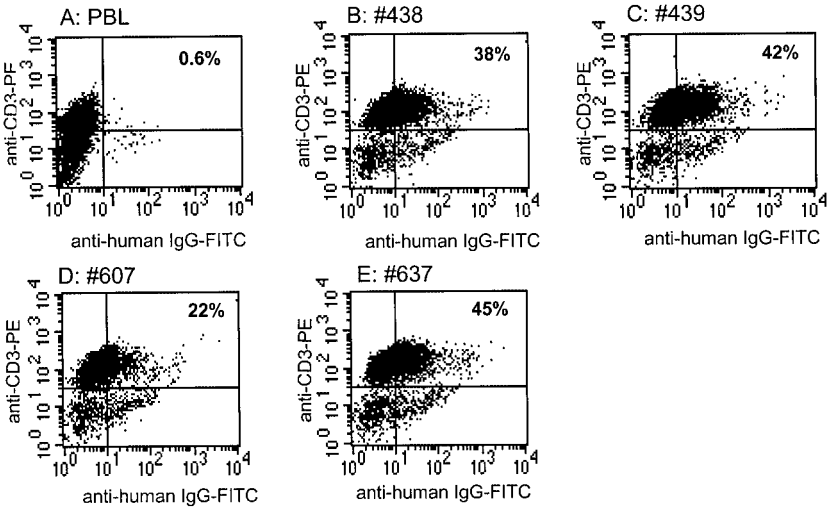

FIGURE 2. Two-color immunofluorescence of anti-CEA receptor-grafted peripheral blood T cells. Nontransduced peripheral blood T cells (*A*) and T cells grafted with the BW431/26-scFv-Fc-γ (438) (*B*), BW431/26-scFv-Fc-ζ (439) (*C*), BW431/26-scFv-Fc-CD28/CD3ζ (607) (*D*), and BW431/26-scFv-Fc-CD28 (637) (*E*) receptor were simultaneously incubated with a PE-conjugated anti-CD3 mAb and a FITC-conjugated anti-human IgG1 Ab and analyzed by flow cytometry.

but no IL-2 was detected after coincubation with $B7^-$ LS174T cells (Fig. 4*A*). Coculture of nontransduced lymphocytes with $B7^+$ and $B7^-$ tumor cells, respectively, did not induce IL-2 secretion, indicating that B7-mediated signaling alone is not sufficient for IL-2 induction. Induction of cytokine secretion by grafted T cells and cytolysis against $B7^+$ and $B7^-$ tumor cells, respectively, are specifically induced by signaling through the BW431/26-scFv-Fc-γ (438) receptor because coculture in presence of the anti-BW431/26 idiotypic mAb BW2064/36 inhibited specific cytokine secretion and tumor cell lysis, whereas incubation with an isotype-matched control Ab (9G10) did not (Fig. 4*B*).

Proliferation of anti-CEA-γR-grafted T cells upon incubation with $B7^+$ CEA^+ tumor cells

To monitor specific proliferation of grafted T cells in the context of B7-CD28 costimulation, we labeled the cell membrane of lymphocytes, grafted with the BW431/26-scFv-Fc-γ (438) immunoreceptor, with the red fluorochrome PKH26, as described in *Materials and Methods*. Labeled lymphocytes were cocultured with B7-positive and $B7^-$ LS174T tumor cells. After 72 h, nonadherent cells were harvested and stained with a FITC-conjugated anti-human IgG1 Ab to monitor cells that express the recombinant immunoreceptor. Flow cytometric analysis revealed that incubation with LS174T cells induced proliferation of immunoreceptor-grafted lymphocytes (Fig. 5). Recombinant receptor-triggered T cell proliferation was substantially enhanced upon coincubation with $B7^+$ LS174T-B7 cells. As controls, lymphocytes with and without specific receptor, respectively, did not proliferate significantly in the absence of CEA^+ tumor cells. Proliferation is predominantly restricted to the cell compartment that expresses the immunoreceptor, indicating that 1) proliferation of grafted lymphocytes is specifically mediated by the anti-CEA receptor, and 2) T cells without CEA-specific immunoreceptor are not induced to proliferate upon incubation with CEA^+ tumor cells, although the tumor cells express B7.

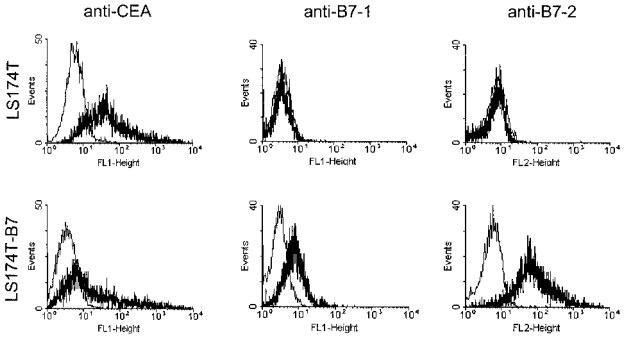

FIGURE 3. Flow cytometric analysis of CEA^+ tumor cells transfected to express B7. LS174T cells (CEA^+) were transfected with pCB/neo DNA to express both B7-1 and B7-2, as described in *Materials and Methods*. Transfected and nontransfected LS174T cells were incubated either with the anti-CEA mAb CEJ065, the FITC-conjugated anti-B7-1 mAb MAB104, the PE-conjugated anti-B7-2 mAb HA5.2B7 (bold lines), or fluorochrome-labeled and nonlabeled isotype-matched control mAbs (thin lines), respectively. Nonconjugated Abs were detected by a FITC-conjugated anti-mouse IgG1 Ab. The cells were analyzed by flow cytometry, and the histograms were overlayed. Note that the CEA expression of B7-transfected LS174T-B7 cells is lower than of LS174T cells. FL1, fluorescence.

Specific activation of $CD4^+$ and $CD8^+$ T cells, grafted with the anti-CEA-Fc-γ receptor, upon cocultivation with B7-expressing tumor cells

To analyze the impact of CD28/B7 costimulation on the immunoreceptor-mediated activation of T cell subsets, we isolated $CD4^+$ and $CD8^+$ T cells from peripheral blood cells by MACS utilizing magnetic beads conjugated with anti-CD4 and anti-CD8 Abs, respectively. By this procedure, we obtained highly enriched $CD4^+$ and $CD8^+$ T cell populations (purity >98% each; data not shown). Both T cell populations were retrovirally transduced to express the anti-CEA BW431/26-scFv-Fc-γ (438) receptor on the cell surface at similar levels (46% transduced $CD4^+$ and 50% $CD8^+$ T cells), as revealed by FACS analysis (Fig. 6). We coincubated receptor-grafted $CD4^+$ and $CD8^+$ T cells in increasing numbers with CEA^+ LS174T and LS174T-B7 and CEA^- A375 tumor cells, respectively, and monitored target cell lysis and IFN-γ and IL-2 secretion. Both receptor-grafted $CD4^+$ and $CD8^+$ T cells specifically lysed CEA^+ tumor cells with high efficiency, whereas nontransduced T cells did not (Fig. 7, *A–C*). Cytolysis of CEA^+ target cells by receptor-grafted $CD4^+$ and $CD8^+$ T cells, respectively, is Ag specific because CEA^- target cells were not lysed, and nontransduced $CD4^+$ and $CD8^+$ T cells without expression of the recombinant anti-CEA-γ receptor did not lyse CEA^+ target cells. Corresponding results were obtained with transduced lymphocytes

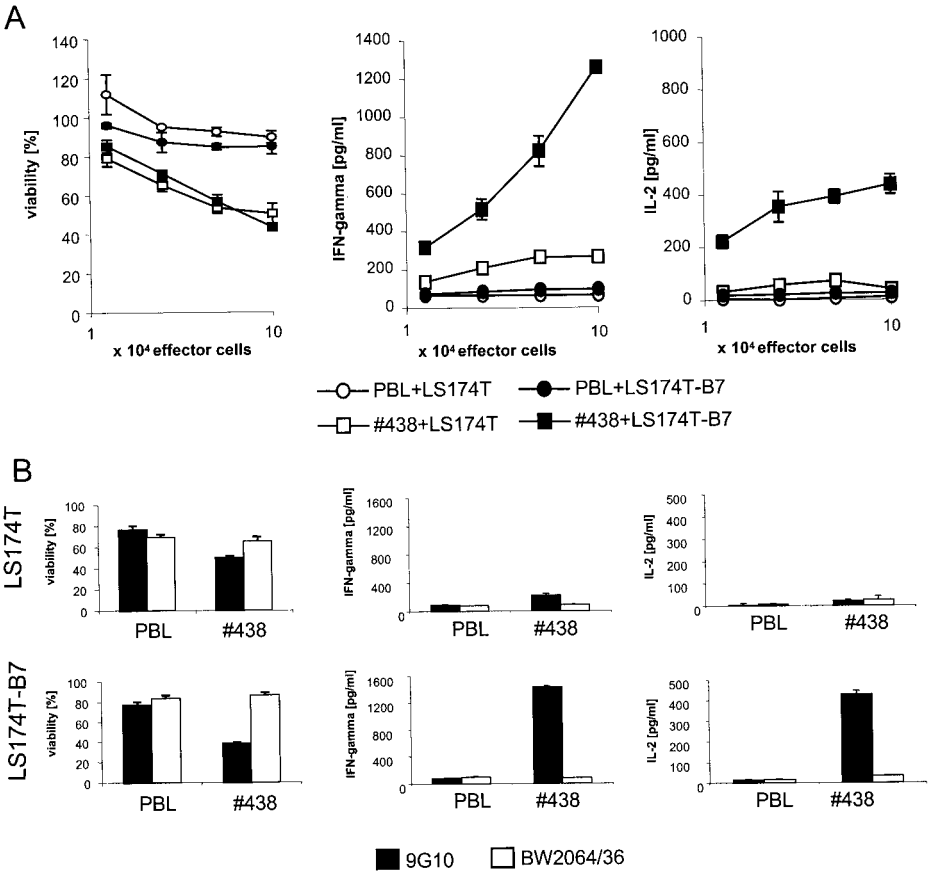

FIGURE 4. Specific activation of T cells grafted with the anti-CEA-γ receptor upon coincubation with B7-transfected CEA$^+$ tumor cells. *A*, Non-transduced T cells (open symbols) and T cells grafted with the BW431/26-scFv-Fc-γ (438) receptor (filled symbols) (1.25–10 × 10^4 cells/well) were cocultivated for 48 h with CEA$^+$ B7$^-$ (LS174T) or CEA$^+$ B7$^+$ (LS174T-B7) tumor cells (each 5 × 10^4 cells/well). *B*, Nontransduced T cells and T cells grafted with the BW431/26-scFv-Fc-γ (438) receptor (5 × 10^4 cells/well) were cocultivated for 48 h with CEA$^+$ B7$^-$ (LS174T; *upper row*) or CEA$^+$ B7$^+$ (LS174T-B7; *lower row*) tumor cells (each 5 × 10^4 cells/well) in the presence of the anti-BW431/26 idiotypic mAb BW2064/36 or the anti-HRS3 idiotypic mAb 9G10 for control (each 2 μg/ml). Viability of target cells and the amounts of IFN-γ and IL-2 secreted by grafted T cells was determined, as described in *Materials and Methods*. The number of BW431/26-scFv-Fc-γ (438) receptor-expressing T cells was 18%. The assay was done in triplicate, and the mean values were determined. The SEM are indicated in the figure.

from different blood donors (not shown). The cell culture supernatants of these experiments were additionally tested by ELISA for the presence of IFN-γ (Fig. 7, *D* and *E*) and IL-2 (Fig. 7, *G–I*). Both receptor-grafted CD4$^+$ and CD8$^+$ T cells secreted high amounts of IFN-γ upon cocultivation with LS174T target cells. IFN-γ secretion of grafted CD4$^+$ T cells was enhanced by B7 costimulation, whereas IFN-γ secretion of grafted CD8$^+$ T cells was not affected by B7 costimulation. In contrast, receptor-mediated IL-2 secretion of grafted CD4$^+$ and CD8$^+$ T cells strictly required B7 costimulation in addition to Ag-mediated receptor signaling because no IL-2 secretion was monitored upon receptor cross-linking in the absence of B7. These data moreover indicate that in receptor-grafted CD4$^+$ and CD8$^+$ T cells, IL-2 secretion is similarly modulated by B7-CD28 costimulation.

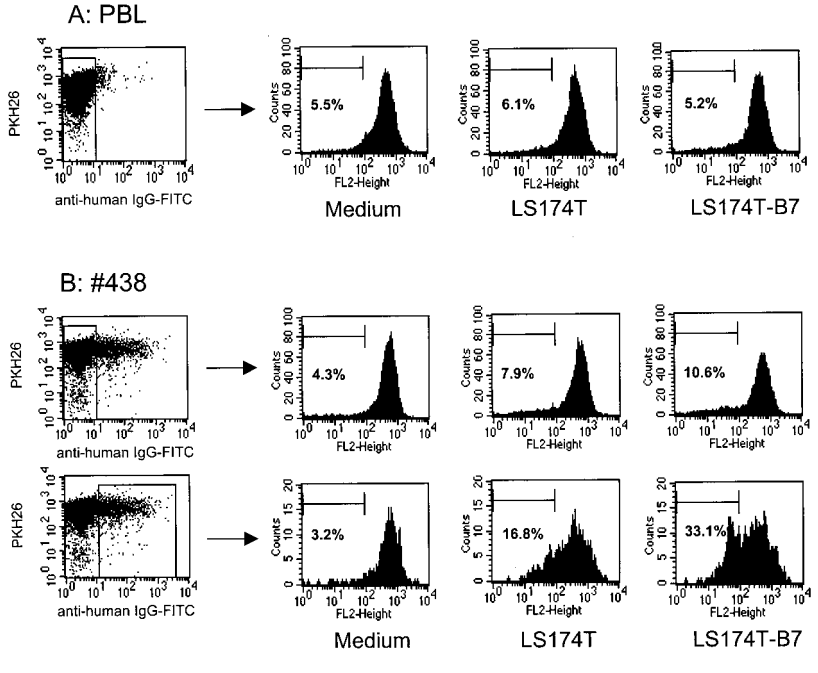

FIGURE 5. Proliferation of T cells grafted with the anti-CEA-γ receptor upon coculture with B7$^+$ and B7$^-$ tumor cells. PBLs (*A*) and lymphocytes grafted with the BW431/26-scFv-Fc-γ (438) receptor (*B*) were labeled with the red fluorochrome PKH26, as described in *Materials and Methods*. Labeled cells (1 × 10^5 cells/well) were cocultivated for 72 h with CEA$^+$B7$^+$ (LS174T-B7) and CEA$^+$B7$^-$ tumor cells (LS174T) (5 × 10^4cells/well). Nonadherent cells were harvested, stained with a FITC-conjugated anti-human IgG1 Ab to monitor receptor-grafted cells, and analyzed by flow cytometry. Gates were set around receptor-expressing and nontransduced lymphocytes, and the number of proliferating cells was determined, as described in *Materials and Methods*. The initial number of receptor-grafted T cells was 18%. FL2, fluorescence.

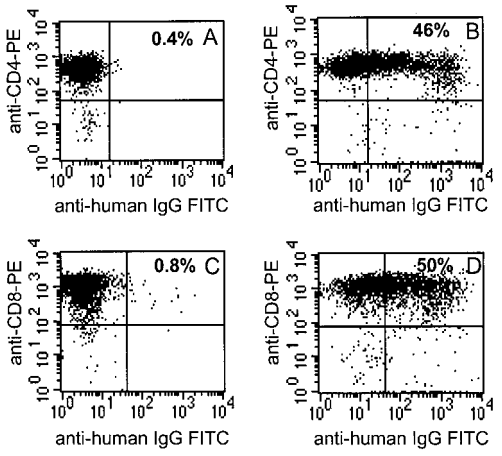

FIGURE 6. Two-color immunofluorescence of purified $CD4^+$ and $CD8^+$ T cells grafted with the anti-CEA-γ receptor. Nontransduced $CD4^+$ (*A*) and $CD8^+$ (*C*) T cells and BW431/26-scFv-Fc-γ (438) receptor-grafted $CD4^+$ (*B*) and $CD8^+$ (*D*) T cells were simultaneously incubated with PE-conjugated anti-CD4 (*A* and *B*) and anti-CD8 (*C* and *D*) mAbs, respectively, and a FITC-conjugated anti-human IgG1 Ab to monitor the grafted receptor. Stained cells were analyzed by flow cytometry.

CD3ζ signaling and CD28 costimulation can be integrated into a single recombinant receptor molecule

The experiments described above indicate that CD28 costimulation is a prerequisite for complete T cell activation via recombinant TCR molecules. We therefore tested whether the costimulatory signal can be delivered additionally via the recombinant receptor independently of signaling by endogeneous CD28. We stimulated receptor-grafted T cells via the recombinant receptor by binding to the immobilized ligand with or without CD28 costimulation and monitored the IFN-γ and IL-2 content in the culture supernatants. Thus, the anti-CD3 mAb OKT3 (2 μg/ml), the anti-CD28 mAb 15E8 (2 μg/ml), the anti-BW431/26 idiotypic mAb BW2064/36 (4 μg/ml) that is directed toward the scFv domain of the receptor, and an isotype-matched IgG1 control mAb (4 μg/ml) were coated alone or in combination onto microtiter plates and were incubated with T cells grafted with the anti-CEA-γ (438), anti-CEA-ζ (439), anti-CEA-CD28/CD3ζ (607), and anti-CEA-CD28 (637) immunoreceptor, respectively. The results of these experiments are summarized in Fig. 8. T cells grafted with the anti-CEA-γ (438), anti-CEA-ζ (439), and anti-CEA-CD28/CD3ζ (607) receptor, respectively, secrete IFN-γ upon stimulation by the receptor ligand BW2064/36 mAb as well as upon stimulation by the anti-CD3 mAb. IFN-γ secretion of anti-CEA-γ (438)- and anti-CEA-ζ (439)-grafted T cells was furthermore enhanced by CD28 costimulation, whereas IFN-γ secretion of anti-CEA-CD28/CD3ζ (607)-grafted T cells was not. Specific IL-2 secretion of anti-CEA-γ (438)- and anti-CEA-ζ (439)-grafted T cells was only observed upon CD28 costimulation in addition to Ag stimulation. In contrast, T cells grafted with the anti-CEA-CD28/CD3ζ (607) receptor secreted high amounts of IL-2 without exogenous CD28 costimulation. IL-2 secretion by these T cells could not further be increased by additional CD28 costimulation. These data indicate that both signals for T cell activation are delivered through the same receptor with a chimeric CD28/CD3ζ signaling domain. T cells grafted with the anti-CEA-CD28 (637) receptor did not secrete significant

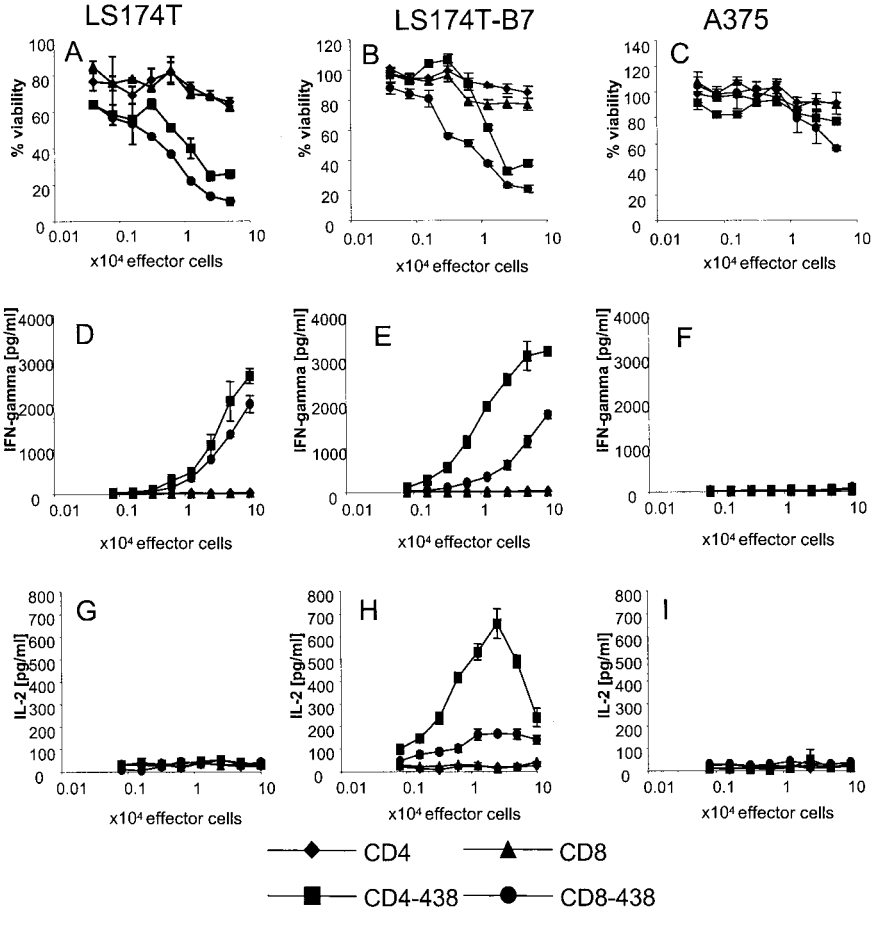

FIGURE 7. Specific activation of purified $CD4^+$ and $CD8^+$ T cells grafted with the anti-CEA-γ receptor upon coincubation with CEA^+ $B7^+$ tumor cells. Purified $CD4^+$ and $CD8^+$ T cells and BW431/26-scFv-Fc-γ (438) receptor-grafted $CD4^+$ and $CD8^+$ T cells ($0.016–5 \times 10^4$ cells/well) were cocultivated for 48 h with CEA^+ $B7^-$ (LS174T), CEA^+ $B7^+$ (LS174T-B7), and CEA^- (A375) tumor cells for control, respectively (each 5×10^4 cells/well). Target cell viability and IFN-γ and IL-2 secretion of grafted T cells were determined, as described in *Materials and Methods*. The number of receptor-grafted $CD4^+$ and $CD8^+$ T cells was 46% and 50%, respectively. The assay was done in triplicate, and the mean values were determined. The SEM are indicated in the figure.

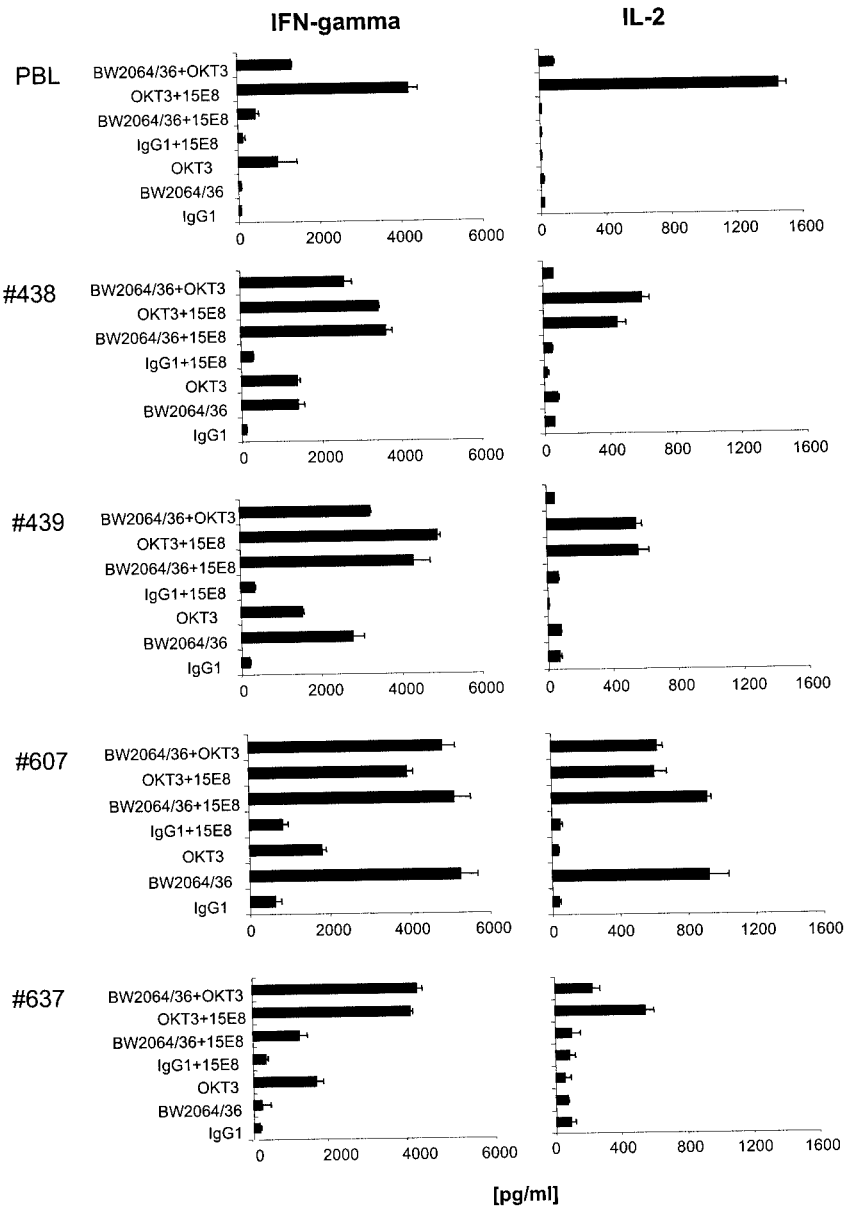

FIGURE 8. Cytokine secretion by peripheral blood T cells grafted with recombinant anti-CEA receptors upon cross-linking and CD28 costimulation by solid-phase bound receptor ligands. Microtiter plates were coated alone or in combination with the anti-BW431/26 idiotypic mAb BW2064/36 (4 μg/ml), a mouse IgG1 mAb (4 μg/ml) as isotype control, the anti-CD3 mAb OKT3 (2 μg/ml), and the anti-CD28 mAb 15E8 (2 μg/ml), respectively. Peripheral blood T cells (1×10^5 cells/ml) were grafted with the BW431/26-scFv-Fc-γ (438), BW431/26-scFv-Fc-ζ (438), BW431/26-scFv-Fc-CD28/CD3ζ (607), and BW431/26-scFv-Fc-CD28 (637) receptor, respectively. Receptor-grafted and nontransduced T cells were incubated for 48 h in coated microtiter plates. The supernatants were harvested and analyzed by ELISA for the secretion of IFN-γ and IL-2, respectively. The numbers of anti-CEA-γ (438), anti-CEA-ζ(438), anti-CEA-CD28/CD3ζ(607), and anti-CEA-CD28 (637) receptor-grafted T cells were 38, 42, 22, and 45%, respectively. The assay was performed in triplicate, and the mean values were determined. The SEM are indicated in the figure.

amounts of IFN-γ or IL-2 upon specific receptor stimulation without the CD3ζ signal. Cytokine secretion of anti-CEA-CD28 (637) receptor-grafted T cells, however, could be enhanced by additionally signaling via the CD3/TCR complex, indicating that the anti-CEA-CD28 (637) receptor can transmit CD28 signaling upon specific receptor cross-linking. In summary, this set of experiments furthermore demonstrates that simultaneous signaling via CD28 and CD3/TCR is required for complete T cell activation.

In a second set of experiments, we cocultivated T cells grafted with the anti-CEA-ζ (439), anti-CEA-CD28/CD3ζ (607), and anti-CEA-CD28 (637) receptor, respectively, with CEA^+ $B7^-$ (LS174T) and CEA^+ $B7^+$ (LS17T-B7) tumor cells and, for control, with CEA^- A375 tumor cells. We recorded specific target cell lysis (Fig. 9*A–C*) and IFN-γ (Fig. 9*D–F*) and IL-2 (Fig. 9*G–I*) secretion of grafted T cells. T cells grafted with the anti-CEA-ζ (439) and anti-CEA-CD28/CD3ζ (607) receptor were highly cytolytic against LS174T cells. The efficiency of cytolysis of LS174T and LS174T-B7 cells was nearly similar, once again demonstrating that the efficiency of Ag-driven cytolysis is independent of CD28 costimulation. Both anti-CEA-ζ (439) and anti-CEA-CD28/CD3ζ (607) receptor-grafted T cells secreted IFN-γ upon specific receptor cross-linking. IFN-γ secretion of anti-CEA-ζ (439) receptor-grafted T cells was furthermore enhanced by B7 expression on LS174T cells, whereas additional B7 costimulation did not enhance IFN-γ secretion of anti-CEA-CD28/CD3ζ (607) receptor-grafted T cells. In contrast to IFN-γ secretion, induction of IL-2 secretion by anti-CEA-ζ (439) receptor-grafted T cells requires CD28 costimulation, whereas anti-CEA-CD28/CD3ζ (607) receptor-grafted T cells secrete high amounts of IL-2 without exogenous CD28 signaling. IL-2 secretion, however, was furthermore enhanced by additional exogenous CD28 costimulation. Remarkably, anti-CEA-CD28/CD3ζ (607) receptor-grafted T cells secreted much more amounts of IL-2 without exogenous CD28 costimulation than anti-CEA-ζ (439) receptor-grafted T cells did upon coculture with $B7^+$ LS174T cells. Nontransduced T cells and anti-CEA-CD28 (637) receptor-grafted T cells neither lysed CEA^+ tumor cells nor secreted IFN-γ and IL-2, respectively, demonstrating again that CD28 signaling alone is not sufficient for complete T cell activation.

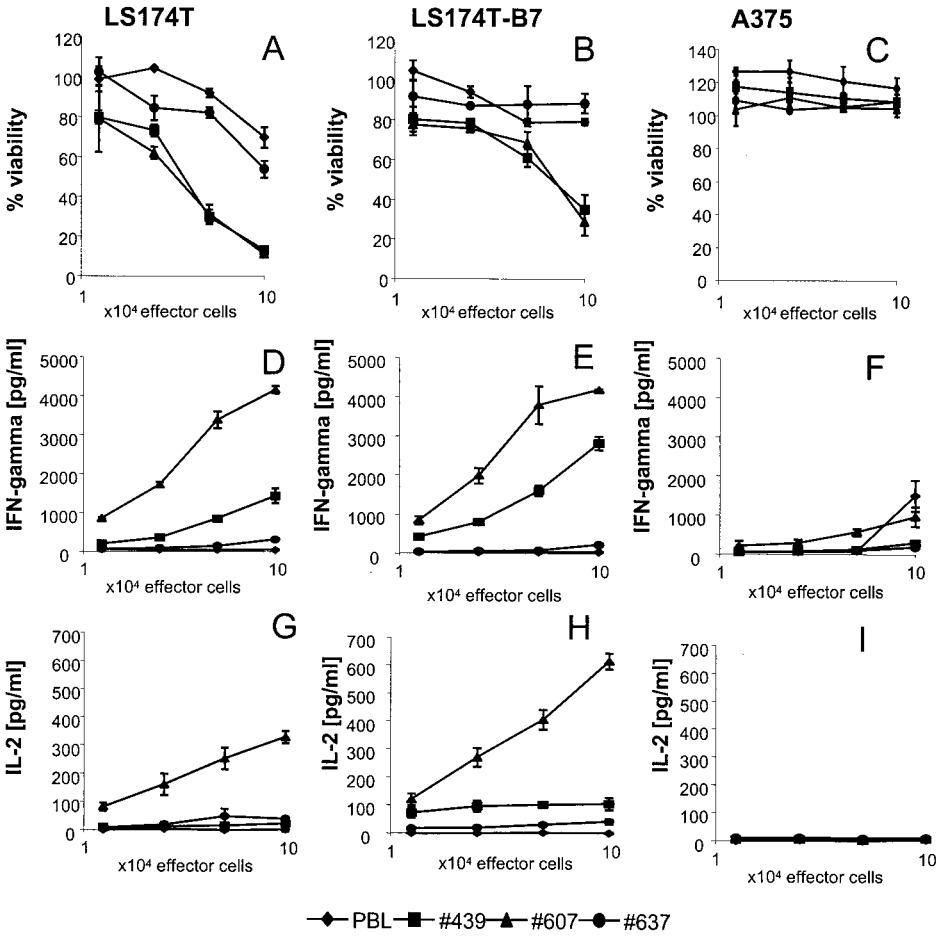

FIGURE 9. Specific activation of receptor-grafted T cells upon coincubation with CEA$^+$ tumor cells. Integration of CD3ζ and CD28 signaling by a chimeric intracellular signaling domain. Nontransduced T cells and T cells grafted with the BW431/26-scFv-Fc-CD28/CD3ζ (607) and, for comparison, with the BW431/26-scFv-Fc-ζ (439) and BW431/26-scFv-Fc-CD28 (637) receptor, respectively (each 1.25–10 $\times$ 10^4 cells/well) were cocultivated for 48 h with CEA$^+$ B7$^-$ (LS174T), CEA$^+$ B7$^+$ (LS174T-B7), or CEA$^-$ (A375) tumor cells for control (each 5 $\times$ 10^4 cells/well). Target cell viability and IFN-γ and IL-2 secretion of grafted T cells were determined, as described in *Materials and Methods*. The numbers of anti-CEA-CD28/CD3ζ, anti-CEA-ζ, and anti-CEA-CD28 receptor-grafted T cells were 22, 42, and 45%, respectively. The assay was done in triplicate, and the mean values were determined. The SEM are indicated in the figure.

Discussion

In this study, we analyzed specific T cell activation via recombinant TCRs in the context of CD28 costimulation using receptor ligands bound to solid phase as well as tumor cells that express the ligand. As a consequence of these analyses, we asked whether CD28 cosignaling can be delivered together with CD3ζ signaling through the same immunoreceptor molecule that harbors the signaling moieties of both CD28 and CD3ζ. We demonstrate in this work that 1) receptor-mediated target cell lysis does not require CD28 costimulation, 2) receptor-mediated induction of cytokine secretion and T cell proliferation is substantially modulated by CD28 costimulation, and 3) CD28 costimulation can be combined with CD3ζ signaling in a single recombinant receptor molecule. These results have substantial impact on the concept of cellular targeting by recombinant receptors: specific target cell lysis by receptor-grafted T cells is independent of CD28 costimulation, allowing efficient cytolysis of B7$^-$, Ag-expressing tumor cells. Other activation parameters such as cytokine secretion and proliferation, however, are uncoupled from the lytic capacity of receptor-grafted T cells and are substantially affected by CD28 costimulation.

The therapeutical efficacy of T cells grafted with immunoreceptors is expected to depend on a long lasting antitumor response. A prolonged antitumor reactivity, however, requires, in addition to short-term tumor cell lysis, sustained proliferation of grafted T cells and secretion of high amounts of IL-2. Since IL-2 plays a key role for T cell proliferation and Th1-based cellular immunity (26), targeting of tumor cells by receptor-grafted T cells without additional CD28 signaling is expected to end in a limited immune response despite high IFN-γ secretion levels. Particularly, the acquisition of additional effector cells at the tumor site, e.g., NK cells, will depend on the presence of IL-2, whose induction requires CD28 costimulation of tumor-specific T cells. Moreover, CD28 costimulation in addition to IL-2 secretion synergistically prevents activation-induced T cell death by up-regulation of the antiapoptotic proteins *bcl*-x$_L$ and *bcl*-2, respectively (27–29). Accordingly, targeting of receptor-grafted T cells without CD28 costimulation is likely to be accompanied by enhanced T cell apoptosis, thus further limiting the therapeutic efficacy. On the other hand, CD28 signaling alone without additional signaling via the endogenous CD3/TCR complex or via a recombinant anti-CEA-ζ and anti-CEA-γ immunoreceptor, respectively, is not sufficient for complete T cell activation, indicating that both signaling pathways must be simultaneously switched on to induce the plethora of T cell activation functions. CD28 costimulatory signals are physiologically delivered by professional APCs, e.g., dendritic cells, to activate CD4$^+$ and CD8$^+$ T cells at the onset of an immune response. Other cell surface molecules with costimulatory activity, e.g., ICAM-1 (30, 31), may only partially substitute B7 costimulation. This will be of physiological significance because the majority of tumor tissues do not express costimulatory molecules of the B7 family. Once activated, tumor-specific CD8$^+$ CTLs are only triggered by peptide-loaded MHC class I molecules or specific tumor-associated Ags on the cell surface. Because the immunoreceptor bypasses MHC molecule-restricted target cell recognition, the recombinant TCR strategy allows also the recruitment of both CD4$^+$ and CD8$^+$ T cell subpopulations for highly efficient target cell lysis (17). However, other cellular effector functions, especially those of grafted CD4$^+$ T cells, will still require efficient costimulation.

To overcome the limitations of appropriate CD28 costimulation in T cell-based adoptive immunotherapy, we generated a recombinant immunoreceptor that harbors the signaling domains of both CD28 and CD3ζ. T cells grafted with this type of immunoreceptor were found to be highly cytolytic and to secrete high amounts of IL-2 upon receptor cross-linking without additional costimulation. These data suggest that both activation pathways are successfully integrated, at least in part, into the anti-CEA-CD28/CD3ζ receptor that combines MHC class I- and class II-independent target cell recognition with dual signaling. We expect that multiple effector functions of both $CD4^+$ and $CD8^+$ T cells engrafted with this type of immunoreceptor will be specifically activated at the tumor site even in the absence of APCs or exogenous costimulation. Accordingly, the combined signaling receptor anti-CEA-Fc-CD28/CD3ζ will be superior to recombinant receptors that activate a single activation pathway only, and is therefore expected to enhance substantially the efficacy of the recombinant receptor approach for use in the cellular immunotherapy of malignant diseases.

Acknowledgments

We thank Dr. R. Bolhuis (Daniel den Hoed Cancer Center, Rotterdam, The Netherlands) for supplying us with the retroviral vector system pSTITCH and pBULLET.

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CORRECTIONS

Andreas Hombach, Anja Wieczarkowiecz, Thomas Marquardt, Claudia Heuser, Loretta Usai, Christoph Pohl, Barbara Seliger, and Hinrich Abken. Tumor-Specific T Cell Activation by Recombinant Immunoreceptors: CD3ζ Signaling and CD28 Costimulation Are Simultaneously Required for Efficient IL-2 Secretion and Can Be Integrated Into One Combined CD28/CD3ζ Signaling Receptor Molecule. *The Journal of Immunology* 2001;167:6123–6131.

In *Materials and Methods*, the oligonucleotides number 1 and 2 given in Table I contain incorrect sequences. The correct table is shown below.

Table I. *Oligonucleotide primers used for the generation of recombinant immunoreceptors*

1-CD28 *Bam*HI-sense:
5′-CTGGATCCCAAATTTTGGGTGCTGGTGGTGGTTG-3′[a]
2-CD28-*Xho*I-antisense:
5′-CTACTCGAGGATCAGGAGCGATAGGCTGCGAA-3′
3-CD28-CD3ζ-antisense:
5′-**GTCTGCGCTCCTGCTGAACTTCACTCTCAGGGAGCGATAGG**CTGCGAAGTC-3′[b]
4-CD28-CD3ζ-sense:
5′-**CCTATCGCTCCCTGAGAGTGAAGTTCAGCAGGAGC**-3′[b]
5-CDζ-*Xho*I-antisense:
5′-CTACTCGAGGATTAGCGAGGGGGCAGGGC-3′
6-Lκ-*Nco*I-sense:
5′-CTACGTACCATGGATTTTCAGGTGCAGATTTTC-3′
7-hIgG1Fc-*Bgl*II-antisense:
5′-CCCACCCAGATCTTTTTTACCCAGAGACAGGGAGAG-GCTCTTCTG-3′

[a]Restriction sites are underlined.
[b]Overlapping sequences are in boldface.

Harnisha Dalwadi, Bo Wei, Matthew Schrage, Tom T. Su, David J. Rawlings, and Jonathan Braun. B Cell Developmental Requirement for the *Gαi2* Gene. *The Journal of Immunology* 2003;170:1707–1715.

Karsten Spicher and Lutz Birnbaumer were inadvertently excluded from the author line. The correct listing is shown below.

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The authors regret that the credit shown below was inadvertently omitted from the footnotes.

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