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CD4$^+$ T Cells Engrafted with a Recombinant Immunoreceptor Efficiently Lyse Target Cells in a MHC Antigen- and Fas-Independent Fashion[1]

Andreas Hombach,* Claudia Heuser,* Thomas Marquardt,* Anja Wieczarkowiecz,* Volker Groneck,* Christoph Pohl,† and Hinrich Abken[2]*

T cells engrafted by a recombinant immunoreceptor with predefined Ag specificity can efficiently lyse Ag-positive target cells in a MHC Ag-independent manner. It is yet unresolved how receptor-grafted CD4$^+$ T cells contribute to MHC Ag-independent target cell lysis. To address this issue, we grafted isolated CD8$^+$ and CD4$^+$ T cells from the peripheral blood with recombinant anti-carcinoembryonic Ag and anti-CD30 receptors, respectively. Cytotoxicity analyses revealed that grafted CD4$^+$ T cells exert cytolysis of Ag-positive target cells with an efficiency similar to that of grafted CD8$^+$ T cells. Lysis by receptor-grafted CD4$^+$ T cells is Ag specific and is inhibited by blocking the target Ag or the Ag binding site of the recombinant receptor. Both Fas-sensitive and Fas-resistant target cells are lysed with equal efficiency, and lysis of Fas-sensitive target cells is not blocked by an anti-Fas ligand Ab, indicating that cytolysis by receptor-grafted CD4$^+$ T cells is independent of the Fas pathway. We conclude that cytolysis by CD4$^+$ T cells equipped with a recombinant immunoreceptor is MHC Ag and Fas independent and likely to be mediated by perforin present in receptor-grafted CD4$^+$ T cells. ***The Journal of Immunology,*** **2001, 167: 1090–1096.**

T cells genetically modified with recombinant immunoreceptors combine the advantages of MHC Ag-independent, Ab-based target recognition with efficient T cell activation upon specific binding to the receptor ligand (1). The extracellular Ag binding domain of this type of a recombinant receptor molecule consists of a single-chain Ab fragment (scFv)[3] derived either from a mAb or a combinatorial Ab library. The intracellular signaling domain is derived from the cytoplasmic part of a membrane-bound receptor capable of inducing cellular activation, e.g., the FcϵRI γ-chain or the CD3 ζ-chain. T cells equipped with recombinant immunoreceptors were demonstrated to induce an Ag-specific immune response in vitro and in vivo upon specific receptor cross-linking by Ag (2–4). Notably, Ag recognition by the Ag binding domain of the immunoreceptor is independent of MHC restriction of the Ag, thereby mediating MHC Ag-independent cytolysis by grafted effector cells. In principle, both CD8$^+$ T cells, restricted to MHC class I Ags, and CD4$^+$ T cells, restricted to MHC class II Ags, can be grafted by recombinant receptors for MHC-independent target cell recognition. After engraftment with recombinant immunoreceptors, however, little is known about the contribution of the CD8$^+$ and CD4$^+$ T cell subset for target cell lysis. Furthermore, the mechanism of CD4$^+$ T cell-mediated target cell lysis as well as the frequency of CD4$^+$ CTL in the peripheral blood is still a matter of controversy. Two main pathways in CTL-mediated cytotoxicity have been described, i.e., granule exocytosis of perforin/granzymes and target cell lysis via the Fas/Fas ligand system. Investigations utilizing Fas mutant *lpr* (5), Fas ligand mutant *gld* (6), and perforin (7)-deficient knockout mice suggest that MHC class I-restricted target cell lysis by murine CD8$^+$ CTLs relies on perforin/granzymes, whereas MHC class II-restricted target cell lysis by murine CD4$^+$ CTLs is predominantly mediated by the Fas/Fas ligand system (8, 9). Accordingly, murine CD8$^+$ T cells engrafted with a recombinant receptor lyse Fas-resistant target cells, whereas CD4$^+$ CTLs did not (10). In contrast to the murine system, the mechanism of cytolysis by human CD4$^+$ T cells is less resolved. Yasukawa et al. (11) recently demonstrated that human CD4$^+$ and CD8$^+$ alloantigen-specific CTL clones and CTL bulk lines, respectively, lyse their target cells predominantly by granule exocytosis and not by the Fas/Fas ligand system. Since human CD4$^+$ and CD8$^+$ CTL clones apparently lyse their targets in a similar fashion, we asked how efficient CD4$^+$ T cells from the peripheral blood can be recruited to MHC Ag- and Fas-independent target cell lysis upon engraftment with recombinant receptors. To address this issue, we retrovirally transduced human CD4$^+$ and CD8$^+$ T cells that were freshly isolated from peripheral blood, to express recombinant receptors with specificity to the carcinoembryonic Ag (CEA) or the CD30 Ag, respectively. Monitoring of each T cell subset with respect to MHC Ag-independent cytolysis of Fas-sensitive and Fas-resistant target cells revealed that receptor-grafted human CD4$^+$ and CD8$^+$ T cells lyse their target cells with similar efficiency and in a Fas-independent fashion.

Materials and Methods

Abs and reagents

The anti-CEA mAb BW431/26, the anti-CD30 mAb HRS3, the anti-idiotypic (anti-Id) mAb 9G10 elicited with mAb HRS3, and the anti-Id mAb BW2064/36 with specificity for the anti-CEA mAb BW431/26 were described earlier (12–14). The hybridoma cell line OKT3, which produces

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[3] 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; anti-Id; anti-idiotypic.

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the anti-CD3 mAb OKT3, was obtained from American Type Culture Collection (ATCC CRL 8001; Manassas, VA). mAbs were affinity purified from murine ascites or hybridoma supernatants using an agarose-immobilized goat anti-mouse IgG1 (Sigma, Deisenhofen, Germany) or a Sepharose (Amersham Pharmacia, Freiburg, Germany)-immobilized goat anti-mouse IgG2a Ab (Southern Biotechnology Associates, Birmingham, AL). The anti-CEA mAb CEJ065 was purchased from Coulter-Immunotech (Hamburg, Germany). The anti-HLA-A, B, C class I mAb W6/32, the anti-HLA-DP, DQ, DR class II mAb CR3/43, 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, FITC-conjugated $F(ab')_2$ anti-mouse IgG1, and PE-conjugated $F(ab')_2$ anti-mouse IgG2a Abs were purchased from Southern Biotechnology Associates. The PE-conjugated anti-human perforin mAb δG9 and a PE-conjugated murine IgG2a control mAb, respectively, the anti-human Fas mAb NOK-1 that neutralizes the cytotoxic activity of the Fas ligand, the anti-human IFN-γ Ab mAb NIB42, and the biotinylated anti-human IFN-γ mAb 4S.B3, respectively, were purchased from BD PharMingen (San Diego, CA). The human Fas ligand fused to the extracellular domain of the murine CD8 was purchased from Ancell (Bayport, MN).

Tumor cell lines

H508 (ATCC CCL 253), SW948 (ATCC CLL 237), and LS174T (ATCC CCL 188) are CEA-expressing colon carcinoma cell lines; Jurkat (ATCC TIB 152) is a CD30-expressing T leukemia cell line; and A375 (ATCC CRL 1619) is a melanoma cell line. The properties of these cell lines are summarized in Table I. The cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS (all Life Technologies, Paisley, U.K.).

Magnetic activated cell sorting (MACS)

$CD4^+$ and $CD8^+$ T cells were isolated from the peripheral blood by MACS using magnetic bead-conjugated anti-CD4 and anti-CD8 mAbs, respectively (both Miltenyi Biotec, 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 with either magnetic beads-conjugated anti-CD4 or anti-CD8 mAbs, according to the manufacturer's recommendations, for 15 min on ice. 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 Biotec). The number of positively enriched $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry as described below. The number of contaminating $CD8^+$ and $CD4^+$ cells was always lower than 2% in the population of enriched $CD4^+$ and $CD8^+$ cells, respectively. MACS-enriched T cells were washed and cultured for 48 h in RPMI 1640 medium supplemented with 10% (v/v) FCS in the presence of IL-2 (400 U/ml; Endogen, Woburn, MA) and OKT3 mAb (100 ng/ml). Cells were transduced as described below.

Generation of chimeric receptors and transduction of $CD4^+$ and $CD8^+$ T cells

The generation of the retroviral expression cassettes for the CEA-specific BW431/26-scFv-Fc-γ and the CD30-specific HRS3-scFv-γ receptor were recently described in detail (15–18). Retroviral vector DNA (pSTITCH-BW431/26-scFv-Fc-γ, pSTITCH-HRS3-scFv-γ) was cotransfected with the retroviral helper plasmid DNAs pHIT60 and pCOLT (15) (each 6 μg DNA) into 293T cells by the calcium phosphate coprecipitation procedure. pHIT60 encodes the MuLV *gag* and *pol* genes; pCOLT encodes the GALV-envelope gene under control of the CMV promotor/enhancer. MACS-isolated $CD4^+$ or $CD8^+$ T cells from the peripheral blood were activated by incubation with OKT3 mAb and IL-2, washed, resuspended in medium with IL-2 (400 U/ml), and cocultivated for 48 h with transiently transfected 293T cells. T cells were harvested, and receptor expression was monitored by flow cytometric analysis.

Table I. *Characteristics of target cell lines*

Cell Line	CEA	CD30	HLA-A, -B, -C	HLA-DP, -DQ, -DR
LS174T	+[a]	−	+	−
H508	+	−	+	−
SW948	+	−	+	−
A375	−	−	+	−
Jurkat	−	+	+	−

[a] Ag expression was monitored by flow cytometric analysis.

Immunofluorescence analysis

BW431/26-scFv-Fc-γ receptor-grafted $CD4^+$ and $CD8^+$ T cells were identified by two-color immunofluorescence utilizing FITC-conjugated anti-human IgG1 (1 μg/ml) and PE-conjugated anti-CD4 or anti-CD8 Abs (each 5 μg/ml). HRS-3-scFv-γ receptor-grafted $CD4^+$ T cells were identified using the HRS3-scFv-specific anti-Id mAb 9G10 (IgG1, 10 μg/ml) and the anti-CD3 mAb OKT3 (IgG2a, 2.5 μg/ml). Bound Abs were detected by FITC-conjugated $F(ab')_2$ anti-mouse IgG1 and PE-conjugated $F(ab')_2$ anti-mouse IgG2a Abs (each 1 μg/ml). Expression of CEA, CD30, and HLA class I and class II molecules, respectively, on target cells was monitored by primary Abs (10 μg/ml) that were detected by a secondary anti-mouse IgG1 FITC Ab (1 μg/ml). Intracellular perforin expression was analyzed by two-color immunofluorescence. Briefly, receptor-grafted $CD4^+$ and $CD8^+$ T cells were incubated for 5 h with GolgiStop (BD PharMingen) according to the manufacturer's recommendations. The cells were washed twice with cold PBS, incubated for 30 min on ice with an anti-human IgG FITC Ab (1 μg/ml), washed, and subsequently fixed and permeabilized using cytofix/cytoperm solution (BD PharMingen) according to the manufacturer's recommendations. After washing with PBS containing 1% (w/v) saponin (BD PharMingen), the cells were incubated with 30 μl of a PE-conjugated mouse anti-human perforin Ab (δG9; BD PharMingen) or an isotype-matched control mAb, respectively, for 30 min on ice, washed, and analyzed by flow cytometry.

Fas-mediated apoptosis of target cells

Sensitivity of target cells to Fas-mediated apoptosis was monitored using recombinant Fas ligand fused to the extracellular part of the murine CD8.

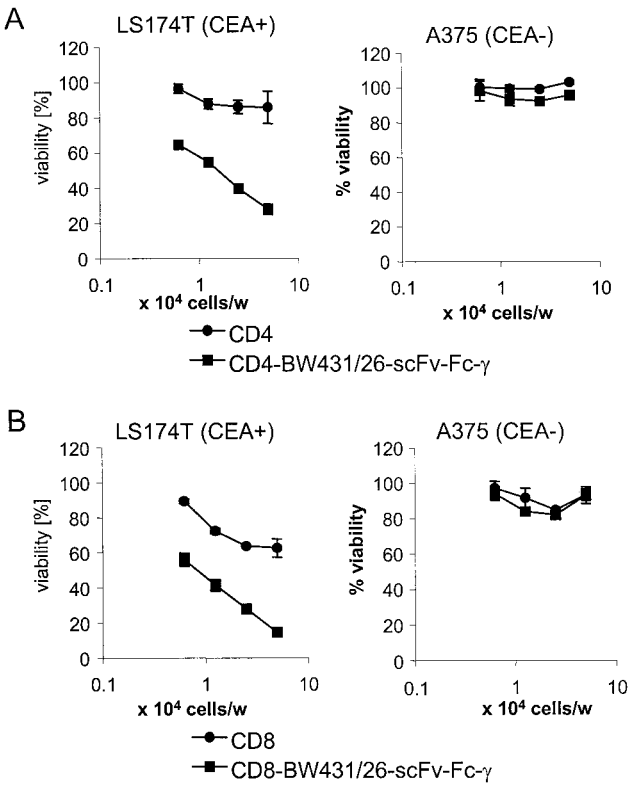

FIGURE 1. Specific cytolysis of CEA^+ target cells by anti-CEA receptor-grafted $CD4^+$ and $CD8^+$ T cells. $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells were grafted with the anti-CEA receptor BW431/26-scFv-Fc-γ, and receptor-grafted and nontransduced T cells (6.25×10^3 to 1×10^5 cells/well) were cocultivated for 48 h with CEA^+ LS174T and for control with CEA^- A375 tumor cells (each 5×10^4 cells/well) in 96-well tissue culture plates. The viability of tumor cells was determined by the XTT assay as described in *Materials and Methods*. The number of receptor-grafted $CD4^+$ and $CD8^+$ T cells was 46.08% and 50.23%, respectively. The assay was done in triplicate.

Briefly, cells were incubated in 96-well microtiter plates with recombinant Fas ligand (2 μg/ml; Ancell) for 24 h. Viability of cells was monitored by a 2,3-bis(2-methoxy-4-nitro-5-sulfonyl)-5[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT)-based colorimetric assay as described below. Specificity of Fas-mediated cell lysis was demonstrated by coincubation with the blocking anti-Fas mAb NOK-1 (10 μg/ml; BD PharMingen).

Stimulation of receptor-grafted T cells and cytokine ELISAs

Receptor-grafted and nontransduced CD4$^+$ and CD8$^+$ T cells (10×10^4–1.25×10^4/well) were cocultivated for 48 h in 96-well round-bottom plates with tumor cells (5×10^4/well). The culture supernatants were analyzed for secretion of IFN-γ, TNF-α, IL-4, and IL-10 by ELISA. 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, Somerville, NJ) as a substrate. TNF-α, IL-4, and IL-10 in the supernatant were detected by cytokine-specific ELISA kits (BD PharMingen; Biosource, Nivelles, Belgium) according to the manufacturer's recommendations. The amount of cytokine was calculated using reference standard curves with known amounts of cytokines.

XTT-based cytotoxicity assay

Specific cytotoxicity of receptor-grafted CD4$^+$ and CD8$^+$ T cells against target cells was monitored by a XTT-based colorimetric assay according to Jost et al. (19). Briefly, receptor-grafted and nontransduced T cells (1×10^5 cells/well) were cocultivated in triplicate in round-bottom microtiter plates with tumor target cells. After 48 h, XTT reagent (1 mg/ml) (Cell Proliferation Kit II; Roche Diagnostics) was added to the cells and incubated for 30–90 min at 37°C. Reduction of XTT to formazan by viable tumor cells was monitored colorimetrically at an OD wavelength of 450 nm and a reference wavelength of 650 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 medium 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: % viability = $(OD_{\text{experimental wells − corresponding number of effector cells}}/OD_{\text{tumor cells without effectors − medium}}) \times 100$.

Specificity of recombinant receptor-mediated cell lysis was demonstrated by coincubation with the anti-Id mAbs BW2064/36 and 9G10, which block the recombinant receptor, or the anti-CD30 mAb HRS3 (each 1.25–40 μg/ml), which blocks the target Ag.

Results

Expression of recombinant immunoreceptors in CD4$^+$ and CD8$^+$ T cells

CD4$^+$ and CD8$^+$ T cells from different donors were isolated 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 (data not shown). Both T cell populations were retrovirally transduced to express the anti-CEA (BW431/26-scFv-Fc-γ) or anti-CD30 (HRS3-scFv-γ) receptor on the cell surface. FACS analyses revealed recombinant receptor expression at similar levels in both T cell populations ranging from ~25–50% (data not shown).

Specific targeting of receptor-grafted CD4$^+$ and CD8$^+$ T cells

To test whether anti-CEA receptor-grafted CD8$^+$ and CD4$^+$ T cells are specifically activated by the recombinant receptor, we cocultivated receptor-grafted CD4$^+$ and CD8$^+$ T cells, respectively, in increasing numbers with LS174T (CEA$^+$) and A375 (CEA$^-$) tumor cells, and monitored target cell lysis by a XTT-based assay, as described in *Materials and Methods*. Receptor-grafted CD4$^+$ and CD8$^+$ T cells were both specifically activated and both lysed CEA$^+$ tumor cells with high efficiency, whereas nontransduced T cells did not (Fig. 1). Notably, CD4$^+$ T cells equipped with the anti-CEA receptor lysed CEA$^+$ tumor cells with similar efficiency as grafted CD8$^+$ T cells. Cytolysis of CEA$^+$ target cells by receptor-grafted CD4$^+$ and CD8$^+$ T cells is Ag specific because CEA$^-$ target cells were not lysed in the assay. Corresponding results were obtained with transduced lymphocytes from different blood donors (data not shown).

We also analyzed the cytokine profile secreted by grafted CD4$^+$ and CD8$^+$ T cells upon cocultivation with CEA$^+$ target cells. The

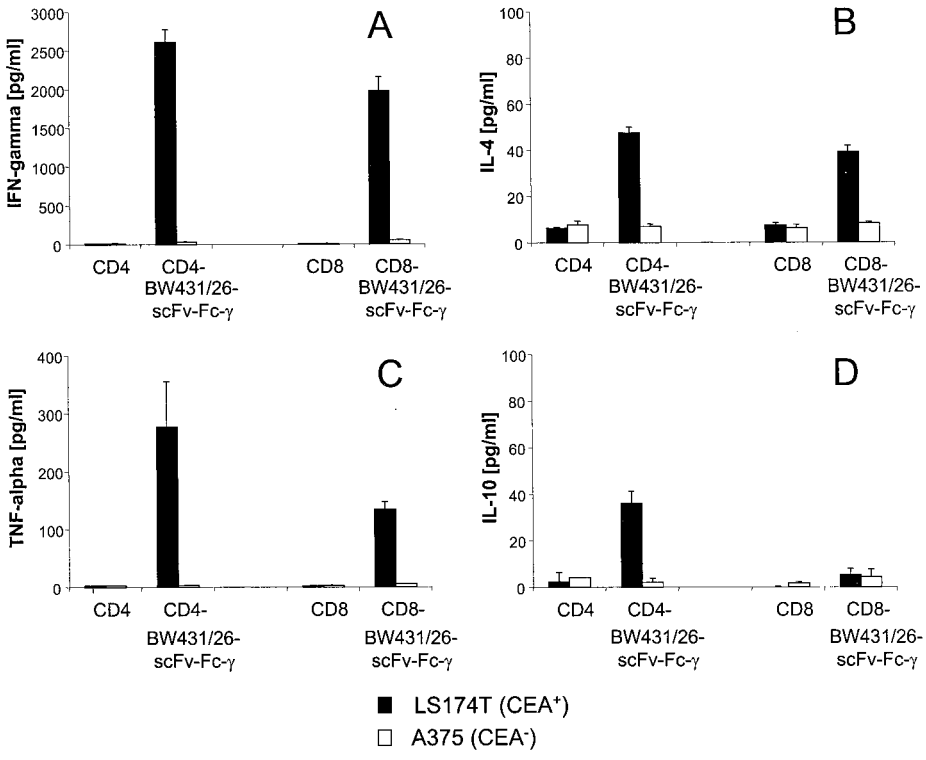

FIGURE 2. Specific activation and cytokine secretion of anti-CEA receptor-grafted CD4$^+$ and CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cells from the peripheral blood were grafted with the anti-CEA receptor BW431/26-scFv-Fc-γ (1×10^5 cells/well), and receptor-grafted and nontransduced T cells were cocultivated for 48 h in 96-well tissue culture plates with CEA$^+$ LS174T (■) and CEA$^-$ A375 (□) tumor cells (each 5×10^4 cells/well). The supernatants were harvested and analyzed by ELISA for the presence of IFN-γ (*A*), IL-4 (*B*), TNF-α (*C*), and IL-10 (*D*), as was described in *Materials and Methods*. The number of receptor-grafted CD4$^+$ and CD8$^+$ T cells was 46.08% and 50.23%, respectively. The assay was performed in triplicate.

cell culture supernatants were tested by ELISA for the presence of IFN-γ, TNF-α, IL-4, and IL-10 (Fig. 2). Both grafted $CD4^+$ and $CD8^+$ T cells secreted high amounts of the predominantly Th1-associated cytokines IFN-γ and TNF-α upon cocultivation with CEA^+ target cells. Receptor-grafted $CD4^+$ T cells, however, additionally secreted detectable amounts of the Th2 cytokines IL-4 and IL-10, wheras grafted $CD8^+$ T cells secreted only low amounts of IL-4, but no IL-10. We conclude that preactivation of T cells with anti-CD3 mAb and IL-2 before retroviral transduction results in grafted $CD4^+$ and $CD8^+$ T cells that secrete predominantly Th1 cytokines in a similar pattern upon Ag-specific receptor cross-linking.

Susceptibility of colorectal tumor cells for Fas-mediated apoptosis

Since murine $CD4^+$ CTL were reported to lyse target cells predominantly via Fas-mediated apoptosis, we asked whether human $CD4^+$ T cells grafted with a recombinant immunoreceptor act in the same way. To monitor the susceptibility to Fas-mediated apoptosis, a set of CEA^+ colorectal tumor cells and, for comparison reasons, Fas-sensitive Jurkat cells were incubated with recombinant Fas ligand, and cytolysis of tumor cells was recorded. As summarized in Fig. 3, LS174T cells are moderately and H508 cells are highly susceptible to Fas-mediated apoptosis, whereas SW948 cells are obviously resistant. As control, Fas ligand was highly cytotoxic toward Jurkat cells. Fas ligand-induced cell death is specific because coincubation with a blocking anti-Fas ligand mAb resulted in complete abrogation of tumor cell lysis, whereas coincubation with an isotype-matched Ab did not (Fig. 3).

Recombinant receptor-mediated cytolysis does not correlate with the susceptibility of target cells to Fas-mediated apoptosis

To investigate the correlation between Fas sensitivity of target cells and cytolysis by receptor-grafted $CD4^+$ T cells, freshly isolated $CD8^+$ and $CD4^+$ T cells were grafted with the anti-CEA and anti-CD30 receptor, respectively, and cocultivated in increasing numbers with Fas-sensitive (H508, Jurkat cells), partially (LS174T cells) and completely (SW948 cells) Fas-resistant target cells. Receptor-mediated cytolysis was monitored as described above. As shown in Figs. 4 and 5, both grafted $CD8^+$ and $CD4^+$ T cells lyse target cells in an Ag-specific manner and with nearly the same efficiency independently of the target cell's susceptibility to Fas-mediated cytolysis. Target cell lysis by grafted T cells is mediated by the grafted receptor because T cells equipped with the anti-CEA receptor lyse CEA^+ target cells (SW948, LS174T, H508), but not CEA^- cells (Jurkat, A375), and T cells equipped with the anti-CD30 receptor lyse $CD30^+$ (Jurkat), but not $CD30^-$ cells (A375, SW948, LS174T, H508). We next analyzed whether receptor-grafted $CD4^+$ T cells use, dependent on the Fas sensitivity of the target cell, different cytolytic pathways for cytolysis. Therefore, anti-CEA and anti-CD30 receptor-grafted $CD4^+$ T cells, respectively, were incubated with different target cell lines (SW948, LS174T, H508, Jurkat) in the presence of the blocking anti-Fas ligand mAb NOK-1 (10 μg/ml) or of the receptor-specific anti-Id mAbs (BW2064/36, 9G10) and the Ag-specific mAb HRS3, respectively. Blocking of the Fas ligand did not alter the efficiency of target cell lysis by receptor-grafted $CD4^+$ T cells independently of the susceptibility of the target cell to Fas-mediated apoptosis (Fig. 6). Cytolysis by $CD4^+$ T cells is mediated by the grafted receptor and is specific because blocking of the Ag binding site of the recombinant receptor with an anti-Id mAb (BW2064/36, 9G10) or blocking of the Ag (HRS3 mAb) inhibited nearly completely target cell lysis (Fig. 6). In addition to this series of blocking experiments, we cocultivated Fas-sensitive H508 cells and anti-CEA receptor-grafted $CD4^+$ T cells in the presence of increasing amounts of up to 40 μg/ml of the blocking anti-Fas ligand mAb NOK-1. The anti-Fas ligand mAb, however, did not inhibit receptor-mediated target cell lysis by receptor-grafted $CD4^+$ T cells even in high concentrations up to 40 μg/ml (data not shown). In summary, we

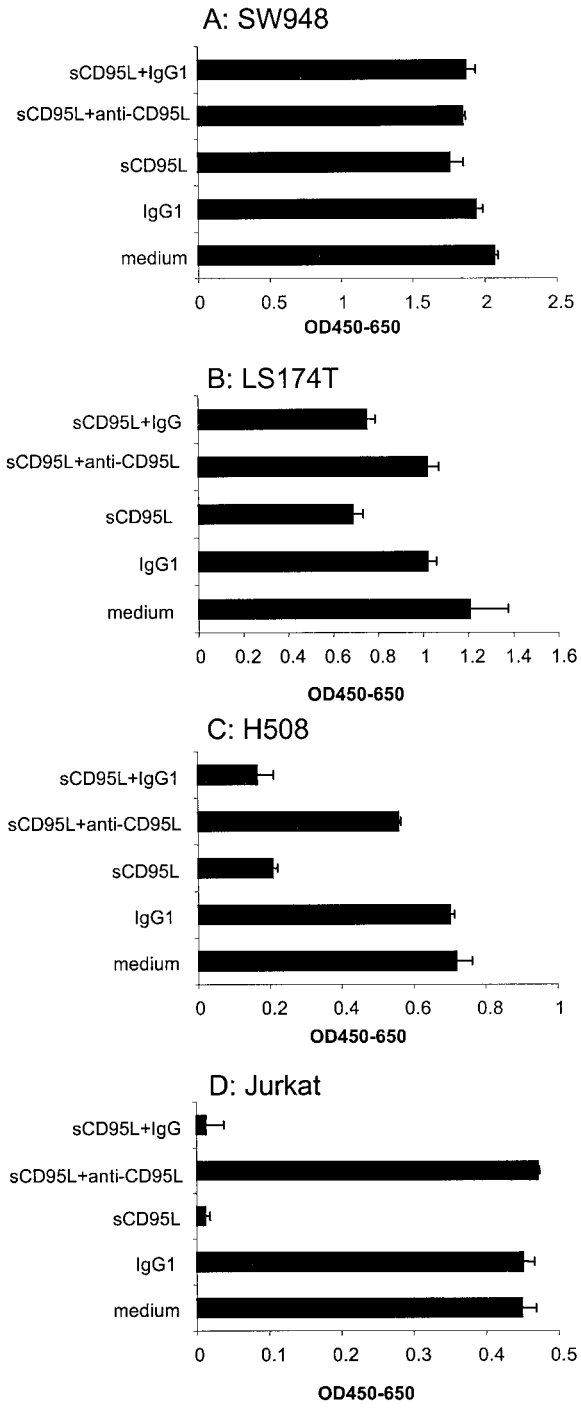

FIGURE 3. Susceptibility of cell lines for Fas ligand-induced cell lysis. Cells of the CEA^+ lines SW948, LS174T, H508, and, as control, of the Jurkat cell line were cultivated for 24 h in 96-well tissue culture plates (4×10^5 cells/well) in medium, in medium plus soluble recombinant Fas ligand (sCD95L-mCD8 protein), and in medium plus an IgG control Ab (each 2 μg/ml). To neutralize the Fas ligand, sCD95-mCD8 protein (2 μg/ml) was coincubated with the blocking anti-CD95L mAb NOK-1 or with an isotype control IgG1 mAb (each 10 μg/ml). The viability of tumor cells was determined by the XTT assay. The assay was done in triplicate.

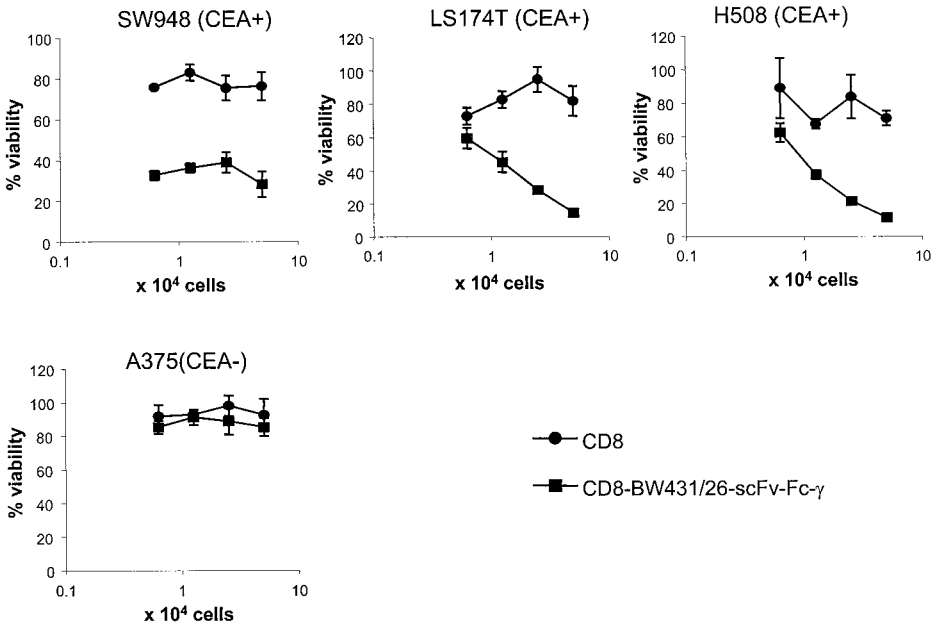

FIGURE 4. Specific cytolysis of Fas-sensitive and Fas-resistant CEA$^+$ cells by anti-CEA receptor-grafted CD8$^+$ T cells. CD8$^+$ T cells were grafted with the anti-CEA receptor BW431/26-scFv-Fc-γ, and receptor-grafted and nontransduced CD8$^+$ T cells (6.25×10^3–1×10^5 cells/well) were cocultivated for 48 h in 96-well tissue culture plates with CEA$^+$ SW948 cells (Fas resistant), LS174T cells (moderate Fas sensitive), H508 cells (highly Fas sensitive), and, for control, with CEA$^-$ A375 cells (each 5×10^4 cells/well). The viability of tumor cells was determined by the XTT assay. The number of receptor-grafted CD8$^+$ T cells was 37.62%. The assay was done in triplicate.

conclude that specific target cell lysis by grafted CD4$^+$ T cells is mediated by recombinant receptor signaling and is independent of Fas.

Expression of perforin in receptor-grafted CD4$^+$ and CD8$^+$ T cells

Since cytotoxicity of recombinant receptor-grafted CD4$^+$ T cells is independent of Fas, we tested whether transduced CD4$^+$ T cells express perforin that, in combination with granzymes, can mediate Fas-independent target cell lysis. Anti-CEA receptor-grafted CD8$^+$ and CD4$^+$ T cells, respectively, and cells of the Hodgkin's lymphoma-derived B cell line HD1236 (20) were intracellularily stained with the PE-conjugated anti-perforin mAb δG9. Receptor-expressing T cells were identified by incubation with a FITC-conjugated Ab directed to the human Fc domain of the receptor. As shown in Fig. 7*A*, both CD4$^+$ and CD8$^+$ T cells express perforin, whereas cells of the B cell-derived line HD1236 do not. We determined the number of CD4$^+$ and CD8$^+$ T cells that simultaneously express perforin and the recombinant receptor by two-color immunofluorescence. Fig. 7*B* demonstrates that the number of receptor-grafted CD4$^+$ T cells with perforin expression is similar to the number of grafted, perforin-positive CD8$^+$ T cells, indicating a significant number of cytolytic T cells within both T cell compartments.

In summary, our data demonstrate that recombinant receptor-grafted human CD4$^+$ T cells from the peripheral blood are highly cytolytic effector cells. These cells are not MHC Ag restricted and lyse their target cells with similar efficiency as their CD8$^+$ T cell counterparts.

Discussion

In this study, we compared the cytolytic potential of human CD4$^+$ and CD8$^+$ T cells from the peripheral blood that were retrovirally engrafted by recombinant immunoreceptors with specificity for

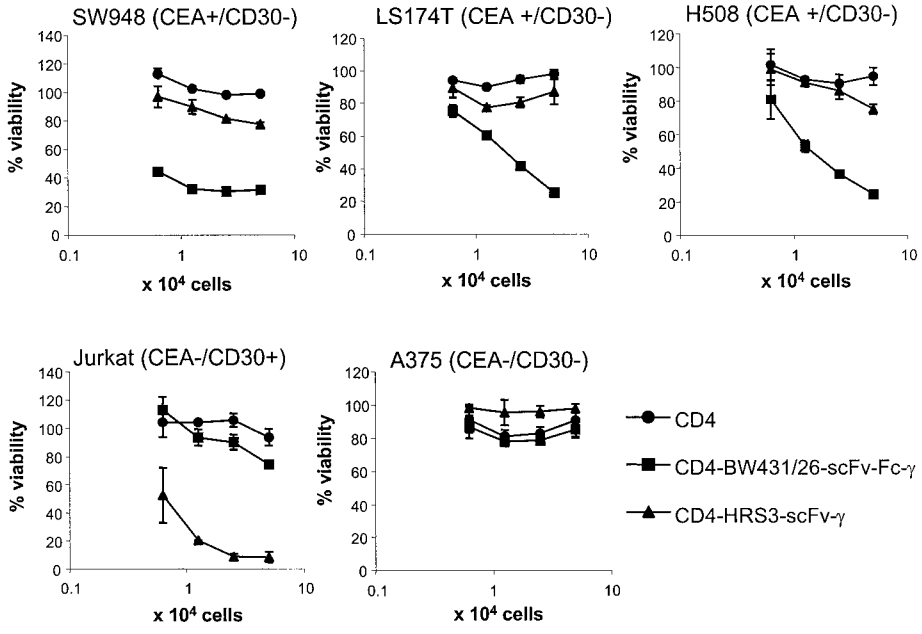

FIGURE 5. Specific cytolysis of Fas-sensitive and Fas-resistant CEA$^+$ and CD30$^+$ target cells by CD4$^+$ T cells grafted with anti-CEA and anti-CD30 receptors. CD4$^+$ T cells were grafted with the anti-CEA receptor BW431/26-scFv-Fc-γ, the anti-CD30 receptor HRS3-scFv-γ, and receptor-grafted and nontransduced CD4$^+$ T cells were cocultivated (6.25×10^3–1×10^5 cells/well) for 48 h in 96-well tissue culture plates with CEA$^+$ CD30$^-$ SW948 cells (Fas resistant), LS174T cells (moderate Fas sensitive), H508 cells (highly Fas sensitive), CD30$^+$ CEA$^-$ Jurkat cells (Fas sensitive), and, for control, with CEA$^-$ CD30$^-$ A375 cells (each 5×10^4 cells/well). The viability of tumor cells was determined by the XTT assay. The number of anti-CEA receptor-grafted and anti-CD30 receptor-grafted CD4$^+$ T cells was 31.9% and 24.66%, respectively. The assay was done in triplicate.

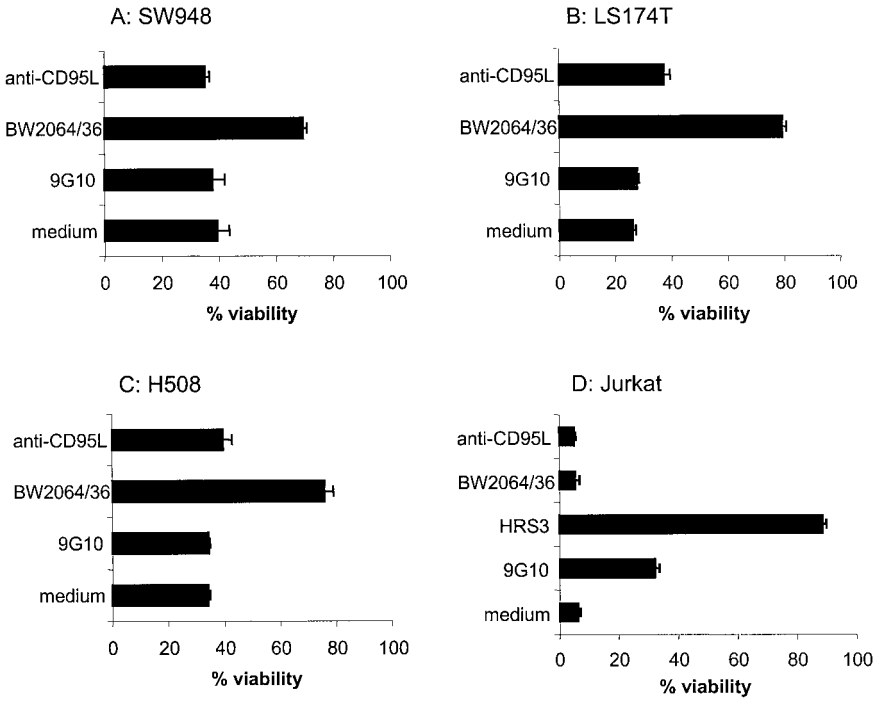

FIGURE 6. Inhibition of target cell lysis by $CD4^+$ T cells grafted with the anti-CEA and anti-CD30 receptor. $CD4^+$ T cells were grafted with the anti-CEA receptor BW431/26-scFv-Fc-γ (*A–C*) and the anti-CD30 receptor HRS3-scFv-γ (*D*) and were cocultivated for 48 h in 96-well tissue culture plates (5×10^4 cells/well) with CEA^+ SW948 cells (*A*), LS174T cells (*B*), H508 cells (*C*), or $CD30^+$ Jurkat cells (*D*) (5×10^4 cells/well) in the presence of the anti-BW431/26-scFv Id mAb BW2064/36, the anti-HRS3-scFv Id mAb 9G10, the anti-Fas ligand mAb NOK-1 (*A–D*), or the anti-CD30 mAb HRS3 (*D*) (each 10 μg/ml). The viability of tumor cells was determined by the XTT assay. The number of anti-CEA and anti-CD30 receptor-grafted $CD4^+$ T cells was 31.9 and 24.66%, respectively. The assay was done in triplicate.

CEA and CD30. These receptors equip grafted T cells, in addition to the specificity of the endogeneous TCR, with the specificity of the recombinant receptor's Ag binding domain and enable grafted T cells to recognize the target Ag in a MHC-independent fashion (1–4). We demonstrate in this study that the MHC-independent Ag recognition enables receptor-grafted $CD8^+$ and, moreover, $CD4^+$ T cells to exert efficient cytolysis of Ag-positive target cells. Notably, the cytolytic activity of grafted $CD4^+$ T cells is highly efficient and comparable with those of grafted $CD8^+$ T cells, indicating that by grafting with a recombinant immunoreceptor both $CD4^+$ and $CD8^+$ T cells can be recruited to a cytolytic, MHC Ag-independent antitumor response with similar efficieny. The set of experiments, on the other hand, also indicates that grafting of $CD8^+$ and $CD4^+$ T cells with this type of immunoreceptor abrogates both MHC class I and class II Ag restriction of target cell recognition. These findings are of general significance since the $CD8^+$ T cell compartment is thought to comprise the majority of cytolytic effector T cells (8). Several reports, however, also describe the presence of cytolytic $CD4^+$ T cells in mice and humans, particularly after stimulation with viral Ags (21). The frequency of cytolytic T cells or their precursors in the peripheral blood and the mechanisms of target cell lysis, however, are still a matter of debate. Our data imply that the proportion of cytolytic $CD4^+$ T cells seems to be nearly similar to those of cytolytic $CD8^+$ T cells because recombinant receptor-grafted $CD4^+$ and $CD8^+$ T cells lyse their target cells with similar efficiency (cf Figs. 1, 4, and 5). Moreover, we demonstrate in this study that the cytolytic response of grafted human $CD4^+$ T cells does not depend on Fas signaling. In contrast, the cytolytic effector functions of murine $CD4^+$ T cells are reported to depend predominantly on Fas signaling (8, 9, 22). Fas-dependent target cell lysis of grafted $CD4^+$ T cells, however, would limit the receptor-mediated, MHC Ag-independent cytolytic response to those cells that are susceptible to Fas-mediated apoptosis. In contrast to the murine system and in accordance with our data, human alloantigen-specific cytotoxic $CD4^+$ T cell clones and oligoclonal T cell cultures were recently demonstrated to lyse MHC class II Ag-expressing target cells in a Fas-independent fashion (23). These experiments used $CD4^+$ T cell clones and oligo-

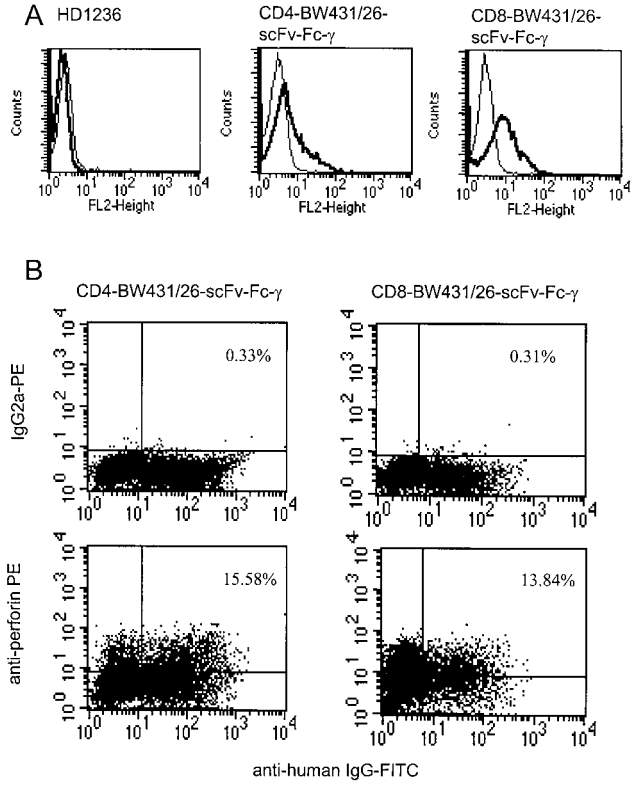

FIGURE 7. Perforin expression in $CD4^+$ and $CD8^+$ T cells from the peripheral blood grafted with the anti-CEA receptor. Anti-CEA receptor-grafted $CD4^+$ and $CD8^+$ T cells and, for control, B cell-derived HD1236 cells were fixed, permeabilized, and incubated with the PE-conjugated anti-perforin mAb δG9 and a PE-conjugated isotype control mAb, respectively, and analyzed by flow cytometry. *A*, Histogram overlays: PE-conjugated IgG2a control mAb (thin lines), PE-conjugated anti-perforin mAb (solid lines). *B*, To identify $CD4^+$ and $CD8^+$ T cells, respectively, that express simultaneously perforin and the recombinant anti-CEA receptor, T cells were additionally incubated with a FITC-conjugated anti-human IgG1-Fc Ab (1 μg/ml) directed to the Fc domain of the grafted receptor.

clonal CD4$^+$ T cell lines generated by long-term cocultivation with allogeneic stimulator cells for several weeks. In this study, we used short-term cultured polyclonal human CD4$^+$ T cells that were obtained from the peripheral blood of several healthy donors and circumvented MHC class II Ag restriction by engraftment with recombinant immunoreceptors. Our results indicate that cytolysis by receptor-grafted, short-term cultured CD4$^+$ T cells is Fas independent because both Fas-sensitive as well as Fas-resistant target cells are lysed with equal efficiency, and an anti-Fas ligand Ab that blocks Fas-mediated apoptosis did not inhibit receptor-mediated cytolysis. We think it likely that Fas-independent target cell lysis of receptor-grafted CD4$^+$ T cells is mediated by perforin because these cells express perforin in substantial amounts, similar to that of CD8$^+$ T cells, and the cytolytic efficiency of grafted CD4$^+$ T cells is very similar to that of receptor-grafted CD8$^+$ T cells. These data, however, do not exclude that, in addition to Fas-independent pathways, receptor-grafted CD4$^+$ T cells may also use the Fas pathway for cytolysis of Fas-sensitive target cells.

In summary, our results indicate that 1) cytolytic CD4$^+$ T cells are present within the human peripheral blood in significant numbers; 2) the MHC class II Ag restriction of CD4$^+$ T cells and the MHC class I Ag restriction of CD8$^+$ T cells can be both circumvented by grafting with an immunoreceptor that harbors a MHC-independent Ag binding domain; and 3) the cytolytic response of CD4$^+$ T cells is independent of functional Fas expression on the target cells. Since the properties of cytolytic CD4$^+$ T cells resemble very much those of CD8$^+$ CTLs, our findings have significant consequences for the cellular immunotherapy using recombinant receptor-grafted T cells. To generate highly efficient effector cell populations for therapeutical reasons, it was recommended to express the recombinant receptor preferentially in purified or highly enriched CD8$^+$ T cells (10). On the basis of our results, we conclude that both MHC class II Ag-restricted CD4$^+$ and MHC class I Ag-restricted CD8$^+$ T cells can be recruited for receptor-mediated, MHC Ag-independent and tumor-specific target cell lysis with nearly the same efficiency.

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