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Costimulatory Signals Through B7.1/CD28 Prevent T Cell Apoptosis During Target Cell Lysis[1]

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Expression of B7 on tumor cells can circumvent T cell tolerance and lead to the generation of tumor cell-specific T cell immunity. The effect of B7 expression on the generation of protective antitumor immunity has been attributed primarily to 1) more efficient T cell activation and 2) better generation of tumor-specific killer T cells. We have investigated the role of costimulation through B7.1 and its receptor, the CD28 molecule, in the generation of allogeneic human CTLs against MCF-7 breast cancer cells. In this setting, we describe how activated CTLs undergo activation-induced cell death upon killing the target cell. Instead of proliferation and clonal expansion, the majority of the CTLs underwent apoptotic cell death. CTL apoptosis could be blocked by 50% when binding of the Fas ligand to its receptor, the CD95 (APO-1/Fas) molecule, was prevented. Fas ligand was detected in the activated T cells, but not in MCF-7 or a panel of other breast cancer cell lines. This excludes an active role for MCF-7 during CTL death and indicates that the CTL apoptosis is due to an autocrine production of the Fas ligand by CTLs. Costimulation of CTLs by retrovirally B7.1-transfected MCF-7 drastically reduced the sensitivity of the CTLs to apoptosis during target contact. Thus, in tumor cell vaccination, B7.1 might play a major role in preventing T cell death by altering T cell susceptibility for apoptosis. *The Journal of Immunology,* **1997, 159: 3808–3815.**

It is generally accepted that T cell activation requires two distinct signals. The first signal is dependent on the ligation of the TCR/CD3 complex and the CD4 and CD8 coreceptors (1). The second signal can be provided by cell surface molecules that mediate essential costimulatory signals, thereby complementing the TCR/CD3-mediated events (2, 3). CD28 is a potent costimulatory molecule, and ligation of CD28 with agonistic Abs or its natural ligands (B7.1 (CD80) and B7.2 (CD86)) synergizes with TCR-mediated signaling to initiate and maintain T cell responses (3, 4). Recently, ligation of CD28 by agonistic Abs was shown to prevent activation-induced cell death (AICD)[3] by apoptosis during activation of resting T cells (5). This was related to the up-regulation of *bcl-xL*, a potent apoptosis-preventing member of the *bcl-2* gene family. Overexpression of *bcl-xL* in Jurkat T cells inhibits both CD3- and CD95 (Fas/APO-1)-mediated apoptosis.

A number of reports have shown that the expression of B7.1 renders tumor cells immunogenic, and the expression of B7.1 in tumor cells has been applied successfully in tumor cell vaccination strategies in animal models (6, 7). Stable expression of B7.1 was demonstrated to induce rejection of tumor cells in a murine syngeneic tumor model compared with that in B7.1-negative tumor cells (6, 7). In addition, protective immunity was induced in those mice transplanted with B7.1-expressing tumor cells. In these systems, the effect of B7.1 was attributed primarily to improved T cell activation and generation of cytotoxic T cells. However, this effect of B7.1 could just as well be explained by the prevention of T cell death upon target cell recognition. In the present study, therefore, we performed tests to ascertain whether T cells undergo apoptosis upon target cell recognition if they are not adequately activated. This was addressed in an alloreactive system based on the generation of allogeneic CTLs against the B7-negative breast cancer cell line MCF-7. We further analyzed the ability of costimulatory signals through anti-CD28 Abs and B7.1 to prevent the activation-induced cell death of CTLs upon target cell recognition.

Materials and Methods

Construction of human B7 gene expression vector and gene transfer experiments

For construction of the TK-CMV vector, a CMV promoter fragment was isolated from tgLS-CMV (20) by PCR using the following primers: 5′-TAG GCG CCG GAA TTC AAG CTT CCA T-3′ and 5′-CAT CGA TGG ATC CCG GGG AGG CTG GAT CGG T-3′. The primers additionally contained cloning sites for *Hin*dIII and *Cla*I, facilitating forced cloning of the PCR product into the tgLS$^+$ HyTK vector (HyTK) (8), resulting in the retroviral vector TK-CMV. The human B7.1 cDNA was obtained by digesting the plasmid pSRI Neo pBJ (2) with *Eco*RI. The 980-bp human B7.1 cDNA fragment was cloned in pBluescript II KS$^+$ *Eco*RI and rescued as a *Bam*HI-*Cla*I fragment. A *Bam*HI site located in the downstream primer used for PCR of the CMV promoter enabled cloning of the human B7.1 fragment into the *Bam*HI-*Cla*I restriction site of TK-CMV, resulting in vector TK-CMV-hB7.1. The vector was transfected into PA317 cells (9) by calcium-phosphate coprecipitation as previously described (10), cells were selected in a hygromycin (500 μg/ml)-containing medium, and clones were established that produce a viral titer equivalent to 5×10^4 hygromycin colonies/ml, respectively. Retroviral infection and determination of the viral titer were performed as previously described (10). The virus-containing supernatant of PA-TK-CMV-hB7.1 was used to infect 10^6 MCF-7 mammary adenocarcinoma cells. After 21 days of selection in hygromycin (500 μg/ml), the cell line MCF-7-B7.1 was established. Mock transfectants (MCF-7 mock) using the PA-TK-CMV control vector were generated and

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[3] Abbreviations used in this paper: AICD, activation-induced cell death; TK, thymidine kinase; FasL, CD95/Fas ligand.

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selected in parallel. Both cell lines were confirmed to be helper virus free by hygromycin resistance mobilization assay after prolonged culture.

Cell culture and preparation of T cells

All cells were maintained in RPMI 1640 (Seromed-Biochrom, Hamburg, Germany), 10% heat-inactivated FCS (Life Technologies, Karlsruhe, Germany), 2 mM L-glutamine (Life Technologies), and penicillin-streptomycin (Seromed-Biochrom). T cells were prepared from peripheral blood by means of Ficoll density gradient centrifugation. B cells were removed by depletion with anti-CD19 MACS magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Adherent cells were removed with use of plastic adherence for 2 h at 37°C. The purity of the T cells was controlled by staining with phycoerythrin-labeled anti-CD3 (Becton Dickinson, Heidelberg, Germany) and analyzed on a FACSort (Becton Dickinson). The purity of the T cells was >94%. T cells were activated in 75-cm^2 culture flasks (Nunc, Copenhagen, Denmark) by immobilized OKT3 (flasks coated overnight at 10 μg/ml Ab in PBS) or irradiated (30 Gy) MCF-7 or MCF-7-B7.1 at a T cell/MCF-7 ratio of 10:1. After 24 h, 30 U of IL-2 (Chiron, Ratingen, Germany) was added. The cytotoxicity of T cells was determined in a standard 4-h ^{51}Cr release assay (11).

Immunofluorescence

B7.1 or B7.2 expression was determined by direct staining of 10^6 cells with phycoerythrin-labeled anti-CD80 or anti-CD86 mAb (Becton Dickinson). Surface fluorescence was determined using a FACSort (Becton Dickinson). Dead cells were excluded by propidium iodide counterstaining.

Measurement of apoptosis

Modified cell cycle analysis. Alloreactive T cells (10^6; activated by OKT3 or allogeneic MCF-7 or MCF-7-B7.1) were exposed to 10^5 irradiated target cells (MCF-7 or MCF-7-B7.1) at various time intervals after primary stimulation. After 24 h the epithelial MCF-7 cells were removed by depletion with epithelial cell-specific HEA125 Ab and immunomagnetic beads (Dynal, Chantilly, VA). The remaining T cells were pelleted, washed with ice-cold PBS in U-form 96-well plates, and gently resuspended in 300 μl of hypotonic fluorochrome solution (50 μg/ml propidium iodide and 0.1 M sodium citrate plus 0.1% Triton X-100) as previously described (12). After overnight incubation at 4°C in the dark, the propidium iodide content of the individual nuclei was measured on a FACSort (Becton Dickinson). Cell debris was excluded by adequately raising the forward scatter threshold. Apoptotic nuclei displayed a decreased DNA content below the G1 peak, paralleled by an increase in the side scatter. Anti-CD95 (anti-APO-1 IgG3 or FII23c $F(ab')_2$ were produced as previously described (21). Anti-B7.1 Ab BB-1 and control IgM were purchased from PharMingen (San Diego, CA). The E:T ratios shown refer to the numbers of initially cultured effector and target cells.

JAM test. Activated T cells were labeled with [^{3}H]TdR as previously described (14). Labeling was controlled by harvesting 10^4 T cells on glass filter plates, and nuclear DNA-bound radioactivity was determined by liquid scintillation counting (usually 10,000–20,000 cpm). Labeled cells (10^4; target cells) were cocultured with unlabeled MCF-7 or MCF-7-B7.1 target cells at increasing E:T ratios. After 24 h the cells were harvested on glass filter plates, and nuclear DNA-bound radioactivity was determined by liquid scintillation counting. Apoptosis decreased the nuclear content of radiolabeled DNA. The percentage of DNA fragmentation as measured for apoptosis was calculated as previously described (14): DNA fragmentation (%) = ((DNA-bound activity before incubation) − (DNA-bound activity after incubation))/(DNA-bound activity before incubation) × 100.

mRNA preparation and Northern blotting

RNA preparation was performed at 4°C by hypotonic lysis (10 mM Tris-HCl, 0.15 M NaCl, 1.5 mM $MgCl_2$, and 0.65% Nonidet P-40) and centrifugation for 5 min at 10,000 × *g*. The supernatant was precipitated with 7 M urea, 0.35 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, and 1% SDS, pH 7.5. An equal volume of phenol/chloroform/isomalyl alcohol (50/48/2%) was added, and the samples were centrifuged at 10,000 × *g* for 20 min. The upper phase was precipitated in ethanol, and the RNA was washed twice with 70% ethanol. Ten micrograms of RNA was loaded onto a 1.2% agarose/1.1% formaldehyde gel and separated electrophoretically. Northern transfer of RNA to nylon membranes was performed by vacuum blotting (Appligene, Heidelberg, Germany). After prehybridization (50% deionized formamide, 0.6 M NaCl, 0.04 M NaH_2PO_4 (pH 7.4), 4 mM EDTA, 1% SDS, and 100 μg/ml herring sperm DNA) at 42°C for 16 h, blots were hybridized with ^{32}P random prime-labeled probes (16). CD95 ligand expression was detected using a 500-bp PCR-cloned fragment (sense, ATG TTT CAG CTC TTC CAC; antisense, AGA GAG AGC TCA GAT ACG) obtained from cDNA of anti-CD3-activated Jurkat T cells. β-Actin was detected with a cloned and sequenced 246-bp fragment obtained by reverse transcription-PCR as previously described (13, 16, 17).

Protein preparation and immunoblotting

Cell lysates were obtained as previously described (13). In brief, 20 mg protein extract/lane was electrophoretically separated using 12% SDS-PAGE. Detection of CD95 ligand protein by immunoblotting was performed using the anti-C20 rabbit antiserum purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After transfer to nitrocellulose and blocking (3% BSA and 1× PBS, overnight at 4°C), filters were incubated with the antiserum (dilution, 1/500) in PBS, 1% BSA, and 0.5% Tween-20). Thereafter, filters were incubated with alkaline phosphatase-conjugated goat anti-rabbit (Dianova, Hamburg, Germany; dilution, 1/7500 in PBS, 1% BSA, and 0.5% Tween-20) for 40 min at room temperature. Bands were visualized using the chromogenic substrates NBT/BCIP (Roth, Karlsruhe, Germany). Equal loading was routinely controlled by Ponceau staining.

Results

CD28 prevents FasL-mediated T cell apoptosis during CTL/target cell contact

Activated T cells have been reported to undergo apoptosis upon restimulation by Ag or agonistic Abs against the TCR/CD3 complex (18–20). We, therefore, asked whether human cytotoxic T cells are triggered for apoptosis upon target cell contact and activation. As a model, we established a system allowing for an assessment of T cell apoptosis in mixed CTL/target cell cultures based on the JAM assay (14). Alloreactive CTLs were generated from purified T cells (contaminating non-T cells, <5%) by culture in the presence of irradiated MCF-7 cells. T cells were maintained in low dose IL-2 (30 U/ml) for 7 days. The CTLs were then tested for cytotoxicity against the breast adenocarcinoma MCF-7 cells in the presence or the absence of anti-CD28 Abs during the CTL/MCF-7 coculture. No significant difference in target cytotoxicity between the CTLs exposed to MCF-7 and those exposed to MCF-7 plus anti-CD28 in a ^{51}Cr release assay was observed (Fig. 1*a*). To test whether the T cells undergo apoptosis during target cell contact and activation, we radiolabeled the T cell DNA with [^{3}H]thymidine and determined the amount of T cell nuclear DNA fragmentation by JAM testing. Surprisingly, T cells incubated with the MCF-7 cells underwent apoptosis, as evidenced by the 60% DNA fragmentation (Fig. 1*b*). In contrast, T cells cultured with MCF-7 in the presence of anti-CD28 Abs showed much lower amounts of DNA fragmentation.

AICD of T cells has been reported to be mediated by autocrine production of the Fas (CD95/APO-1) ligand (21, 22). To investigate whether the apoptosis of CTLs upon exposure to the target cells is mediated by activation-induced production of the FasL, we performed a functional experiment in which we added anti-APO-1 IgG3 $F(ab')_2$ to the culture. These $F(ab')_2$ of the anti-APO-1 anti-CD95 Ab (23) prevent binding of the FasL to the CD95 (Fas/APO-1) Ag (21). This experiment was performed at a fixed E:T ratio of 10:1. The T cells were activated with irradiated MCF-7 during the primary stimulation and restimulated with irradiated MCF-7 (secondary stimulation) on day 7. Apoptosis was measured on the single-cell level (12) after 24 h of culture of T cells in the presence of MCF-7 target cells. The remaining epithelial cells were removed by the epithelial cell-specific mAb HEA125 and immunomagnetic beads. Eighty percent of the T cells showed hypodiploid (apoptotic) nuclei compared with 20% in the medium control. The addition of anti-APO-1 $F(ab')_2$ to block FasL binding to CD95 inhibited the apoptosis of CTLs upon MCF-7 target cell contact by approximately 50% (Fig. 2*a*). Isotype-matched $F(ab')_2$ did not inhibit T cell apoptosis. Northern blot analysis of the activated T cells showed that T cells do not express FasL before

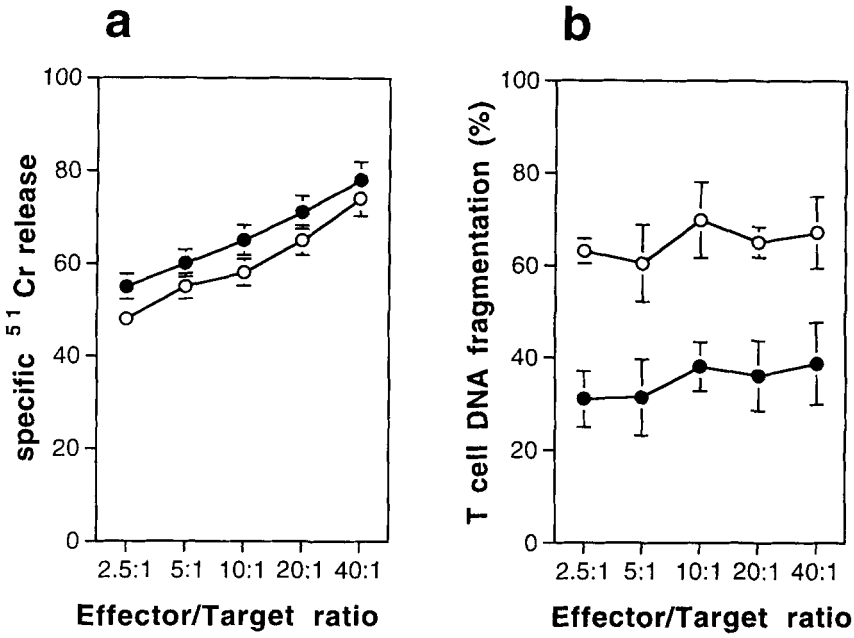


FIGURE 1. AICD in alloreactive human CTLs and protection by anti-CD28 Abs. *a*, ^{51}Cr release assay of MCF-7. Peripheral T cells were activated by irradiated MCF-7 cells and maintained for 7 days in IL-2-containing medium (30 U/ml). Alloreactive cytotoxicity against MCF-7 breast adenocarcinoma targets was determined in a standard 4-h ^{51}Cr release assay in the absence (white circles) or the presence of anti-CD28 (clone 15E8 at 1 μg/ml; black circles) at different E:T ratios. *b*, JAM assay for T cell AICD. T cells were isolated, activated, and maintained as described in *a*. After 6 days of culture, the T cells were labeled overnight with [^{3}H]TdR. The radiolabeled T cells were then cultured for 24 h in the absence or the presence of MCF-7 target cells. DNA-bound radioactivity was measured by liquid scintillation counting, and the percentage of DNA fragmentation, as a measure of apoptosis, was calculated as previously described (14). White circles indicate T cells plus MCF-7; black circles indicate T cells plus MCF-7 plus anti-CD28 (clone 15E8 at 1 μg/ml). Data are given as the mean ± SD of a typical experiment performed in triplicate.

secondary stimulation with MCF-7, but up-regulate the 1.6-kb FasL mRNA upon target cell contact (Fig. 2*b*). No FasL mRNA could be detected in MCF-7 before or after the addition of T cells (Fig. 2*b*). Exposure of the alloreactive T cells to MCF-7-B7.1 transfectants did not change FasL mRNA expression by the T cells compared with that by T cells exposed to MCF-7 mock transfectants (Fig. 2*c*). A similar pattern of FasL expression was found in Western blot analysis (Fig. 2*d*), which confirms that no FasL is expressed by the MCF-7 cells. FasL protein was expressed in T cells upon exposure to the target cells. Costimulation through anti-CD28 or B7.1-expressing MCF-7 cells did not alter FasL expression in the T cells. Anti-CD28 mAb alone did not induce FasL protein in the alloreactive CTLs (Fig. 2*d*).

Effect of B7.1 on CTL apoptosis

In the aforementioned experiments, we showed that costimulation with anti-CD28 Abs inhibits AICD of CTLs upon target cell contact. To further investigate the role of costimulation of CTLs through CD28, we constructed a retroviral vector containing human B7.1 under control of a CMV promoter (TK-CMV-hB7.1; Fig. 3*a*). The PA-TK-CMV-hB7.1 virus was obtained after packaging in PA317 cells and was used to transduce MCF-7 breast cancer cells with the human B7.1 gene. After 21 days of selection in hygromycin (500 μg/ml), the cell line MCF-7-B7.1 was established. Surface staining with anti-B7.1 Abs confirmed that all cells expressed B7.1 (Fig. 3*b*), whereas no B7.1 could be detected on mock transfectants transduced with PA-TK-CMV (MCF-7-mock).

T cell activation by the transduced cell lines was assessed by generating CTLs. T cells were activated using irradiated MCF-7-B7.1 or MCF-7-mock, maintained for 7 days in the presence of IL-2 (30 U/ml). Cytotoxicity, measured by ^{51}Cr release of MCF-7-mock or MCF-7-B7.1 target cells, showed that in this situation MCF-7-mock cells were lysed to a similar extent as MCF-7-B7.1 transfectants. Nevertheless, B7.1-expressing target cells were lysed slightly more efficient by the alloreactive T cells (Fig. 4, *a* and *b*, black symbols). Generation of CTLs using irradiated MCF-7-B7.1 led to a consistently higher cytotoxic activity upon incubation with MCF-7-mock or MCF-7-B7.1 target cells (Fig. 4*b*) compared with primary activation with MCF-7 (Fig. 4*a*). To exclude that differences in MHC class I expression were responsible for this increased induction of allo-CTLs by the B7.1-expressing MCF-7, we analyzed MHC class I expression by immunofluorescence and flow cytometry. Both MCF-7 and MCF-7-B7.1 cells highly expressed MHC class I, as detected by staining with mAb W6/32 (MHC class I monomorphic), without a significant difference between the parental and the B7.1 transduced line (data not shown).

The alloreactive T cells that were generated with irradiated MCF-7 were analyzed for apoptosis induction during target cell lysis. This was performed at the single-cell level by measuring the DNA content of isolated T cell nuclei (Fig. 5). T cells were cultured for 24 h at an E:T ratio of 10:1. The survival of the T cells was not decreased by medium alone, anti-CD28, IgM-control Abs, or anti-B7.1 Abs. Exposure to irradiated MCF-7-mock targets induced apoptosis in 72% of the T cells. This could be decreased to 25% when the T cells were incubated with MCF-7-B7.1 target cells or to 43% when MCF-7-mock in the presence of anti-CD28 Abs was employed as activator. Addition of the control-IgM Ab did not affect this rescue from apoptosis. Incubation of the T cells with MCF-7-B7.1 target cells in the presence of anti-B7.1 Abs, however, almost completely counteracted the rescue from apoptosis and induced apoptosis in 66% of the T cells. These experiments demonstrate that T cell apoptosis during secondary activation upon target cell contact is prevented by CD28/B7-mediated costimulation.

Costimulation during primary stimulation prevents T cell apoptosis upon restimulation

In the above experiments, we showed that costimulation protects CTLs from apoptosis during secondary stimulation, e.g., target cell contact. One would expect, however, that such a protective effect would be provided during the primary activation as well. To test whether costimulation during primary activation protects the T cells from apoptosis during secondary stimulation (target cell contact), we performed the following experiments: T cells were activated by irradiated MCF-7-mock or MCF-7-B7.1 (Fig. 6). The

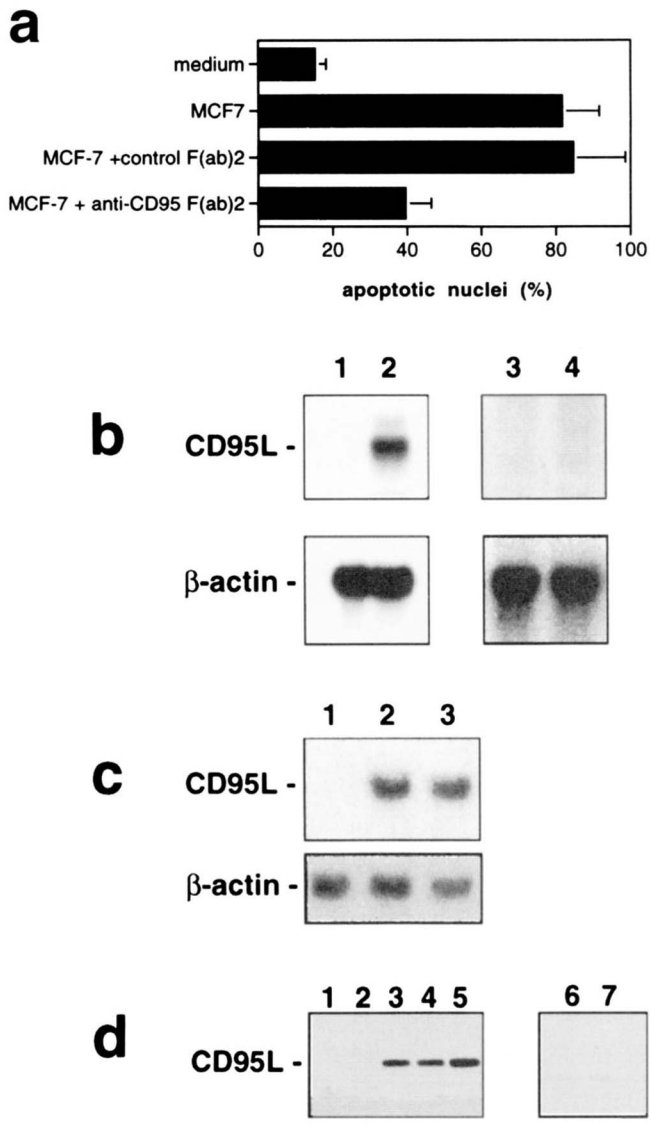


FIGURE 2. FasL expression and function. *a*, Blocking experiment. Peripheral T cells were activated by irradiated MCF-7 cells and were maintained for 7 days in IL-2-containing medium (30 U/ml). The activated T cells (10^6) were then incubated for 24 h in medium alone (no MCF-7) or with irradiated MCF-7 cells at an E:T ratio of 10:1. The incubation was performed in the absence or the presence of anti-CD95 (anti-APO-1 IgG3) $F(ab')_2$ or isotype-matched control $F(ab')_2$ (FII23c) (23). After incubation, the remaining epithelial cells were removed from the coculture by immunomagnetic depletion using the epithelial cell-specific HEA125 mAb. T cell apoptosis was measured on the single-cell level as previously described (12). Data are given as the mean ± SD of a typical experiment performed in triplicate. *b*, Northern blot analysis for FasL expression. T cells were activated as described in *a*. RNA was extracted from the T cells or from MCF-7 cells before and after 2 h of coculture. After the coculture, contaminating MCF-7 cells were depleted with immunomagnetic beads to obtain pure T cells. Contaminating T cells were removed with anti-CD3 mAb OKT3 and immunomagnetic beads to obtain pure MCF-7 cells. CD95 ligand expression was detected using a 500-bp ^{32}P random prime-labeled PCR-cloned fragment that hybridizes to a 1.6-kb fragment. β-Actin was detected with a cloned 246-bp fragment obtained by reverse transcription-PCR as previously described (16). *Lane 1*, Activated T cells before restimulation; *lane 2*, activated T cells after coculture with MCF-7; *lane 3*, MCF-7 before exposure to T cells; *lane 4*, MCF-7 after coculture with T cells. *c*, Northern blot analysis for FasL expression in costimulated T cells. T cells were activated as described in *a*. *Lane 1*, Alloreactive T cells before restimulation; *lane 2*, alloreactive T cells after coculture with MCF-7; *lane 3*, T cells after coculture with MCF-7-B7.1. *d*, Western blot analysis for FasL expression. CTLs were exposed for 12 h to the target cells before protein extracts were prepared. *Lane 1*, Alloreactive T cells before restimulation; *lane 2*, addition of anti-CD28 mAb (15E8; 1 μg/ml); *lane 3*, T cells after coculture with MCF-7; *lane 4*, T cells after coculture with MCF-7 plus anti-CD28 mAb; *lane 5*, T cells after coculture with MCF-7-B7.1; *lane 6*, MCF-7 after coculture with T cells; *lane 7*, MCF-7-B7.1 after coculture with T cells.

activated T cells were then reexposed to MCF-7-mock target cells or MCF-7-B7.1. Those T cells that were activated by MCF-7-mock and restimulated after 3 days (Fig. 6*a*) with MCF-7-mock target cells showed induction of T cell apoptosis. In contrast, T cells exposed to MCF-7-B7.1 during primary stimulation were completely protected from apoptosis upon secondary stimulation with MCF-7-mock (Fig. 6*a*). Specifically, 40% of the T cells activated with MCF-7-mock died by target-induced apoptosis during restimulation compared with only 10.6% of the T cells activated by MCF-7-B7.1 (Fig. 6*a*). However, expression of B7.1 on the MCF-7 target cells protected the MCF-7-mock-activated T cells from target-induced apoptosis (Fig. 6*a*). Anti-CD28 mAb also protected T cells from target-induced apoptosis (data not shown). Nevertheless, in most experiments protection from T cell apoptosis by B7.1 was stronger than that by anti-CD28 Abs during secondary stimulation. After 7 days of culture (Fig. 6*b*), the T cells costimulated by the B7.1 Ag during the primary stimulation again showed lower apoptosis induction than T cells activated by MCF-7-mock alone. Eighty-five percent of the T cells activated with MCF-7-mock, but only 59% of the T cells activated by MCF-7-B7.1, underwent apoptosis upon restimulation with MCF-7-mock (Fig. 6*b*). Surprisingly, the protection from target-induced apoptosis was lower than the effect seen during the secondary stimulation in Fig. 6*a*, which took place 3 days after the primary stimulation. Ten days after primary activation (Fig. 6*c*), no difference in the rate of target-induced apoptosis was seen between T cells stimulated with MCF-7-mock and those stimulated with MCF-7-B7.1 during the primary activation. Anti-CD28 Abs added during primary stimulation provided no protection against T cell apoptosis after a 7- or 10-day culture period (data not shown). In contrast, costimulation by B7.1 on the MCF-7-B7.1 (or by anti-CD28; data not shown) during the secondary stimulation consistently protected CTLs from apoptosis triggered by target cell contact (Fig. 6, *a–c*).

Discussion

T cell activation is initiated by signals transmitted through the TCR/signal transduction complex (signal 1). Other costimulatory receptor-ligand interactions between T cell and APC are needed, however, for complete activation (signal 2). Signaling by Ag on APC, which provide the Ag signal in combination with costimulation, leads to activation, proliferation, and differentiation. Signaling through the TCR alone can lead to anergy (1) or AICD (24). Costimulation by CD28-mediated signals affects a wide variety of T cell activation parameters (reviewed in Refs. 3 and 4). Recently, costimulation with anti-CD28 Abs was shown to protect activated primary T cells and Jurkat T ALL cells from CD3- and CD95-mediated apoptosis (5).

This prompted us to investigate the question of whether primed cytotoxic T cells undergo apoptosis upon secondary stimulation during target cell contact. In additional experiments, we addressed the role of costimulation 1) by anti-CD28 Abs and 2) by means of artificially introduced B7.1. We used the generation of allogeneic T cells by activation with either immobilized anti-CD3 Abs or

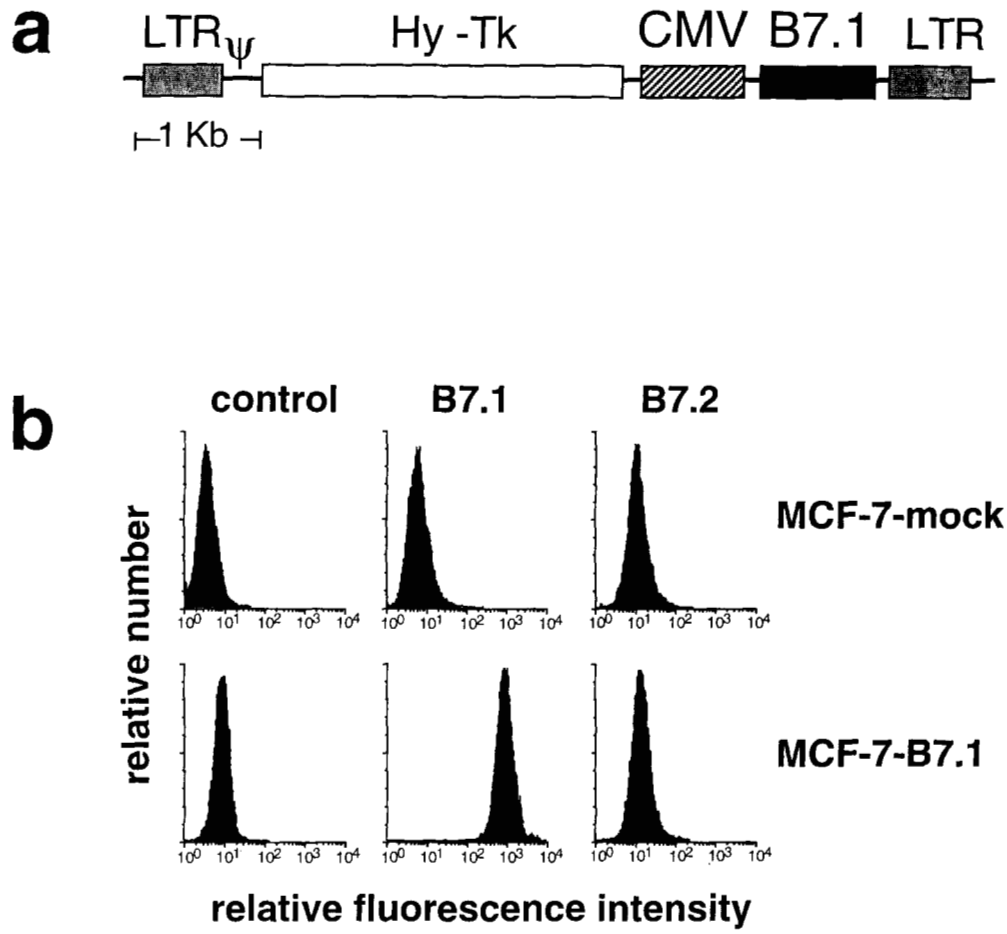
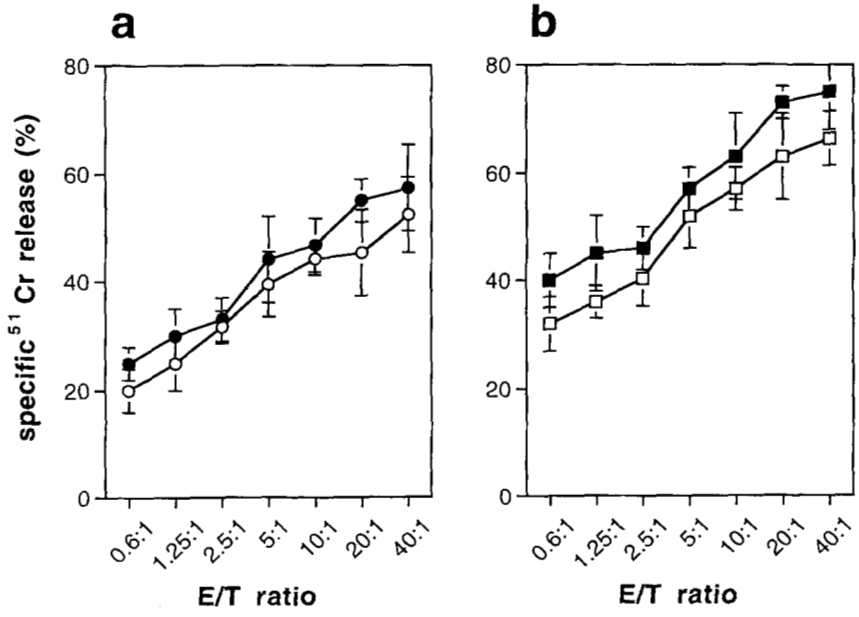


FIGURE 3. Retroviral gene transfer in MCF-7. *a*, Vector map of TK-CMV-hB7.1 retroviral vector. *b*, Surface expression of B7.1 on MCF-7 mock transfectants and MCF-7-B7.1. Cells were stained with phycoerythrin-labeled anti-CD80 mAb, anti-CD86, or isotype-matched control Abs, and surface immunofluorescence was measured on a FACSort flow cytometer. Dead cells were excluded by propidium iodide counterstaining.

FIGURE 4. Cytotoxicity of alloreactive CTL against MCF-7 transfectants. Peripheral T cells were activated during the primary stimulation with irradiated MCF-7-mock (*a*) or MCF-7-B7.1 (*b*) at an E:T ratio of 10:1 and were maintained for 7 days in IL-2-containing medium (30 U/ml). Cytotoxicity against MCF-7-mock or MCF-7-B7.1 breast adenocarcinoma targets during secondary stimulation was determined in a standard 4-h ^{51}Cr release assay. *a*, T cells were activated during the primary stimulation by irradiated MCF-7-mock (day 0). White circles indicate MCF-7-mock as target cell; black circles indicate MCF-7-B7.1 as target cell. *b*, T cells were activated during the primary stimulation by irradiated MCF-7-B7.1 (day 0). White squares indicate MCF-7 as target cell; black squares indicate MCF-7-B7.1 as target cell. Data are given as the mean ± SD of a typical experiment performed in triplicate.



irradiated MCF-7 breast adenocarcinoma cells as a model system. We found that a substantial proportion of the activated CTLs dies by apoptosis upon target cell coculture. The rate of T cell apoptosis upon target cell contact was not significantly altered at different E:T ratios. The reason for this dose-independency remains unclear. A possible explanation for this striking lack of an increase or a decrease in T cell AICD at higher E:T ratios could be that activation of the T cells at low E:T ratios is already adequate and not

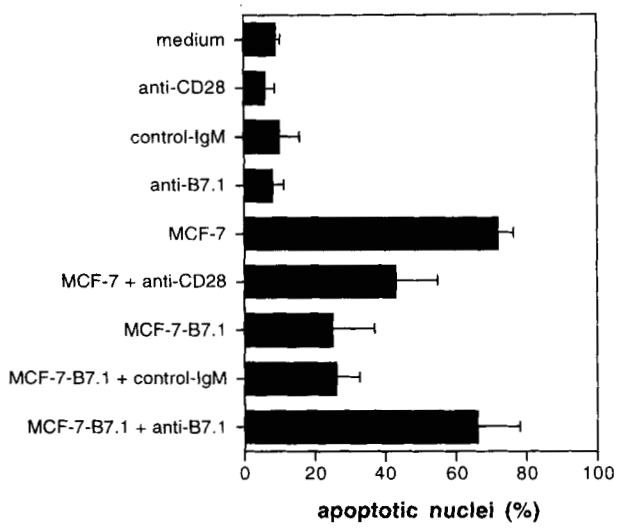


FIGURE 5. Effect of B7.1 on CTL apoptosis. Alloreactive T cells were generated by activation of peripheral blood T cells with irradiated MCF-7-mock (E:T ratio, 10:1) and culture in the presence of IL-2 (30 U/ml) for 7 days. T cells were then cultured for 24 h with medium alone, anti-CD28 (15E8; 1 μg/ml), IgM control mAb (G155–228; 10 μg/ml), or anti-B7.1 (BB-1; 10 μg/ml) in the absence or the presence of irradiated MCF-7-mock or MCF-7-B7.1 at an E:T ratio of 10:1. After incubation, the remaining epithelial cells were removed from the coculture by immunomagnetic depletion. T cell apoptosis was measured on the single-cell level as previously described (12). Data are given as the mean ± SD of a typical experiment performed in triplicate.

dramatically altered by an increase in the E:T ratio. In addition, we performed experiments in which activated T cells were targeted to MCF-7 cells or immature B cell-leukemia cells by the use of CD3/MUC1- or CD3/CD19-bispecific Abs. These Abs form a bridge between activated T cell and tumor target cell that leads to strong cross-linking of CD3 on the T cell, activates the T cell, and induces target cell lysis. In analogy to the results obtained with alloreactive CTLs in the experiments described above, we found that the CD3 cross-linking induced by the bispecific Abs leads to AICD of the targeted CTLs (our manuscript in preparation).

In previous reports, production of CD95/FasL has been shown to mediate activation-induced death of T cells (21, 22). CD95/Fas is a surface receptor that is induced in lymphoid cells by activation (25, 26). In recent studies, expression of FasL has been reported at immunologically privileged sites and on a colon carcinoma cell line (27–30). This expression of FasL was shown to induce apoptosis of CD95/Fas-sensitive T cells, suggesting that FasL plays a role in the maintenance of peripheral tolerance and the deletion of tumor-specific T cells. We, therefore, investigated whether the target cell-induced T cell apoptosis in our systems could be attributed to the production of FasL by the target cells (27) or whether this was due to the CTLs themselves, as in other cases of AICD (21). FasL production by the MCF-7 target cells could be excluded. Furthermore, no FasL could be detected by Northern blot analysis in a panel of breast cancer cell lines and in normal or malignant breast tissue specimens (data not shown). FasL was produced by the activated T cells only after restimulation with the MCF-7 target cells. The addition of blocking anti-CD95 $F(ab')_2$, which prevent CD95/Fas receptor-ligand interaction (21), drastically reduced T cell AICD upon restimulation with MCF-7 cells. The protection from AICD by the $F(ab')_2$ was in the range of 50%, which is line with the literature (21, 22) and suggests, as previously described (31), that CD95/Fas-independent mechanisms contribute to T cell AICD. One such pathway can be activated through TNF/TNF receptor 2 signaling (31, 32). Costimulation by agonistic anti-CD28 Ab or expression of B7.1 on the target cell inhibited CTL AICD during restimulation with the target cell. These results differ from earlier observations showing an independence of CD95/Fas-mediated apoptosis from costimulation via CD28 (33, 34). In our system, we observed, however, that B7 expression or, to a slightly lesser extent anti-CD28 mAb, protected from AICD of CTLs during target cell contact. This AICD differs from anti-CD3-induced T cell death in that anti-CD3 activated T cells are resistant for anti-CD3-triggered cell death during the first days after activation. They start to acquire sensitivity for CD3-triggered AICD after 6 days of culture, and significant amounts of T cells can be induced to AICD via CD3 cross-linking after 10 to 14 days of culture (18). In contrast, AICD of CTLs during exposure to their target cells was detectable as early as 3 days after the primary stimulation, and a large proportion of the CTLs died upon contact of B7-negative target cells when the T cells had been activated and maintained in IL-2-supplemented medium for 7 days. Furthermore, we employed not only anti-CD28 mAb, but also B7-expressing target cells to modulate CTL survival. The MCF-7-B7.1 transfectants expressed large amounts of B7.1 in a membrane-bound form that provides strong cross-linking of CD28 or CTLA-4 on the T cell. One might speculate whether these differences in T cell activation could change the outcome of signaling events involved in T cell activation through costimulation and apoptosis, such as activation of the phosphatidylinositol 3-kinase or the mitogen-activated protein kinase cascade (35). In addition, different events seem to be involved in T cell AICD. Apart from FasL production leading to autocrine and paracrine T cell death, triggering of TNF receptor 2 participates in T cell AICD (31, 32). This can explain why blocking of FasL binding inhibited veto apoptosis of the CTLs only by 50%. TNF receptor 2-mediated apoptosis appears to be more prominent during the early days after primary T cell activation (32), while CD95/Fas sensitivity is low during this phase and increases with time (36, 37). Thus, while our data show that a substantial proportion of CTL death during target contact is mediated by FasL, the protection mediated via B7/CD28 costimulation could in addition be mediated by interference with the TNF death pathway. Our data show that an alteration of FasL expression by costimulation is not involved in protection from CTL apoptosis, since B7.1 expression on the target cells did not decrease FasL expression at the RNA or the protein level in activated T cells. Expression of B7.1 on the target cells furthermore slightly increased the lytic activity of the CTLs. Both better T cell activation and an improved adhesion via B7/CD28 or B7/CTLA-4 (38) during the killing could be responsible for such a phenomenon. The improved generation of CTLs upon primary stimulation with B7.1-expressing stimulator cells was not a consequence of alterations in MHC class I expression and is in line with earlier findings that B7 costimulation of T cells increases cytotoxicity (3).

Even more importantly, costimulation of the T cells during primary stimulation also conferred protection from CTL AICD upon target cell restimulation. However, the T cells were only protected from AICD upon restimulation by costimulation during the primary activation for a limited period of time. Significant protection was only achieved up to day 3 after the primary stimulation. When the restimulation was conducted 7 or 10 days after the primary stimulation, the primarily costimulated T cells became as sensitive to CTL AICD during secondary stimulation as those that were not costimulated. This suggests that to protect them from AICD upon restimulation, activated T cells might require frequent restimulation in the presence of costimulatory molecules such as B7.1. In these experiments, late activated (days 7–10) T cells showed an

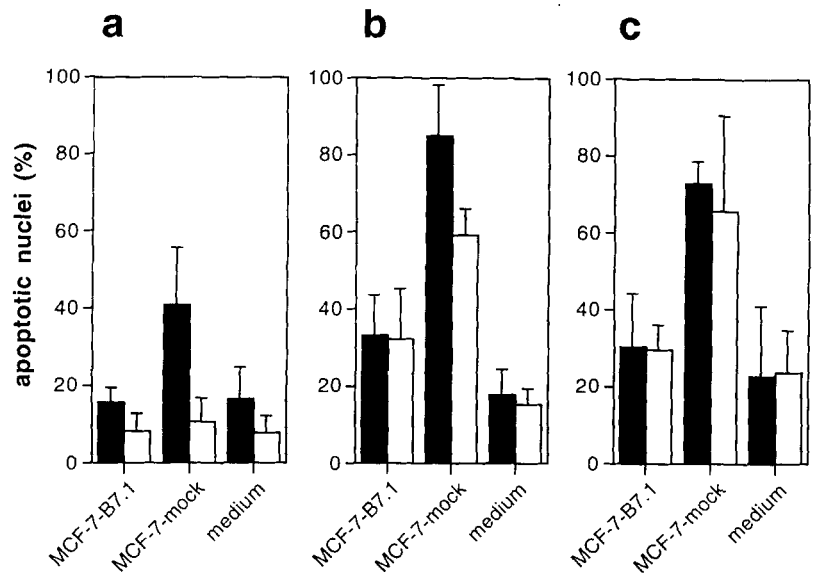


FIGURE 6. Effects of B7.1 and anti-CD28 during primary and secondary T cell stimulation. Alloreactive T cells were generated by activation of peripheral blood T cells with irradiated MCF-7-B7.1 or MCF-7-mock (E:T ratio, 10:1; primary stimulation). Cells were cultured in the presence of IL-2 (30 U/ml) for 3 to 10 days. T cells were then restimulated for 24 h with medium alone, MCF-7-mock, or MCF-7-B7.1 (E:T ratio, 10:1; secondary stimulation). After incubation, the remaining epithelial cells were removed from the coculture by immunomagnetic depletion. T cell apoptosis was measured on the single-cell level. Black bars indicate primary activation with MCF-7-mock transfectants; white bars indicate primary activation with MCF-7-B7.1 transfectants. Culture was performed in IL-2-containing medium for 3 days (*a*), 7 days (*b*), or 10 days (*c*) before restimulation with target cells. Data are given as the mean ± SD of a typical experiment performed in triplicate.

increased sensitivity for AICD compared with T cells examined early (day 3) after the primary activation. This is in line with earlier reports demonstrating that sensitivity for both CD95/Fas- and CD3/TCR-mediated apoptosis increases with time after primary T cell activation (18, 36, 37).

Assuming that such a mechanism is active in vivo, this phenomenon might be a mechanism for maintaining immune tolerance. The absence of costimulation would lead to the generation of highly apoptosis-sensitive T cells. A T cell activated by a nonprofessional APC without costimulation during the primary activation would thus be able to kill once and then be deleted by AICD. It is a known fact, however, that CTLs can cycle, at least in vitro, from one target to another and trigger multiple target kills. Thus, one CTL can release a target and program one or multiple others for lysis. Our data, therefore, do not exclude that multiple inadequate stimulations are required for a single CTL to undergo AICD. Nevertheless, such a T cell response would be abortive and self-limiting, and could lead to immune tolerance. Thus, the CTL that was activated by signal 1 alone could lyse some target cells, but would finally die by AICD unless it was rescued from apoptosis by costimulatory signals. This is of potential importance for immune-based therapeutic strategies. T cell activation by Ag alone could preferentially lead to activation and priming for subsequent T cell deletion (or anergy induction). Hence, the efficiency of B7.1 in combination with cytokines that we and others have observed in tumor vaccination (3, 6, 7) might be explained not only by a more efficient T cell activation but also by the prevention of in vivo T cell AICD by the costimulatory signals.

Finally, our finding that costimulation protects CTLs from AICD during target cell contact clarifies earlier data concerning CTL death. These studies had focused on backward, bystander, and veto killing of CTLs (11, 19, 39–41). ^{51}Cr release of CTLs after 4 to 8 h was used as read-out, whereas we used a longer incubation period of 24 h and apoptosis-specific assay systems based on the detection of DNA fragmentation to assess T cell apoptosis. As described, T cell AICD is relatively slow, and DNA fragmentation can be detected 6 to 8 h after activation (21). Loss of membrane integrity leading to ^{51}Cr release, however, is a late event in the apoptotic process. In the majority of studies examining CTL death, murine spleen cells or EBV-transformed B cells are used as Ag-presenting target cells (11, 19, 39–41). These cells, however, express large amounts of costimulatory molecules such as B7.1. Thus, the CTLs received not only signal 1, but also the apoptosis-preventing signal 2. Our finding that costimulation prevents T cell AICD in peripheral blood-derived alloreactive T cell lines might, therefore, be a general phenomenon, with broad implications for immune homeostasis.

Given the potential importance of this novel regulatory concept, we would suggest using the term veto apoptosis. The veto apoptosis can be mediated by expression of FasL or other molecules on the tolerance-inducing target cell (27–30, 42) or, as we describe, by autocrine production of FasL in the absence of the costimulatory signal 2. In analogy to the well-established veto phenomenon (39–41), the T cell activation by signal 1 alone would lead to activation and induce sensitivity for AICD upon target cell contact. Such an apoptosis-inducing signal could be delivered by every (MHC-positive) body cell capable of Ag presentation. This would represent a more general model for the regulation of CTL survival and immune homeostasis, which extends the concept of veto suppression (39–41).

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