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CD70-Driven Costimulation Induces Survival or Fas-Mediated Apoptosis of T Cells Depending on Antigenic Load

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Apoptosis plays an essential role in the removal of activated CD8 T cells that are no longer required during or postinfection. The Bim-dependent intrinsic pathway of apoptosis removes effector CD8 T cells upon clearance of viral infection, which is driven by withdrawal of growth factors. Binding of Fas ligand to Fas mediates activation-induced T cell death in vitro and cooperates with Bim to eliminate CD8 T cells during chronic infection in vivo, but it is less clear how this pathway of apoptosis is initiated. In this study, we show that the costimulatory TNFR CD27 provides a dual trigger that can enhance survival of CD8 T cells, but also removal of activated CD8 T cells through Fas-driven apoptosis. Using in vitro stimulation assays of murine T cells with cognate peptide, we show that CD27 increases T cell survival after stimulation with low doses of Ag, whereas CD27 induces Fas-driven T cell apoptosis after stimulation with high doses of Ag. In vivo, the impact of constitutive CD70-driven stimulation on the accumulation of memory and effector CD8 T cells is limited by Fas-driven apoptosis. Furthermore, introduction of CD70 signaling during acute infection with influenza virus induces Fas-dependent elimination of influenza-specific CD8 T cells. These findings suggest that CD27 suppresses its costimulatory effects on T cell survival through activation of Fas-driven T cell apoptosis to maintain T cell homeostasis during infection. The Journal of Immunology, 2012, 188: 4256–4267.

Upon recognition of pathogen-derived peptides on APCs, Ag-specific CD8 T cells within the naive CD8 T cell pool expand and differentiate into effector CD8 T cells to combat infection. After pathogen clearance, the effector CD8 T cell population contracts, and only a fraction survives to become memory CD8 T cells that provide enhanced protection upon reencounter with the pathogen (1, 2). Two separate signaling pathways, the intrinsic and extrinsic pathways of apoptosis, are involved in mediating T cell death to control the population size of the T cell pool during infection (3). The intrinsic pathway is mediated by members of the Bcl-2 family of proteins, which control integrity of the mitochondrial outer membrane and regulate release of cytochrome c from the mitochondrial intermembrane space to activate caspase-9 and induce formation of the apoptosome (4, 5). Prosurvival molecules such as Bcl-2 and Bel-XI antagonize proapoptotic molecules such as Bim and Puma, and the balance of these molecules determines whether mitochondrial outer membrane integrity is maintained or lost and survival or apoptosis ensues, respectively (4, 5). The other major pathway of apoptosis, known as the extrinsic pathway, is mediated by death domain-containing molecules such as Fas and TRAIL receptor, which can directly activate caspase-8 and induce apoptosis upon ligand binding (6).

The importance of cell death in CD8 T cell responses has been shown using mice lacking or overexpressing proteins of the intrinsic and extrinsic pathway. Molecules such as Bim and Puma play an instrumental role in executing apoptosis under conditions of limiting growth factors. Mice lacking these proapoptotic proteins display strongly enlarged T cell responses during acute infection and delayed contraction upon pathogenic clearance (7, 8). The extrinsic cell death pathway mediated by Fas ligand (FasL), and Fas does not respond to growth factor withdrawal. In contrast, in vitro and in vivo experiments using Fas-deficient lpr mice have shown that Fas induces apoptosis of activated T cells upon repetitive antigenic stimulation in a process called activation-induced cell death (9, 10). More recently, using mice deficient for both Fas and Bim, several groups have shown that the intrinsic and extrinsic pathways cooperate to control the size of CD8 T cell responses during some but not all viral infection models (11–13). After HSV infection, contraction depends exclusively on Bim, whereas contraction after murine γ-herpes virus infection requires both Fas- and Bim-dependent pathways. Therefore, it was proposed that Bim controls contraction upon acute infection in contrast to Fas that is predominantly involved in eliminating cells during chronic responses (11).

CD27 is a member of the TNFR superfamily that upon binding of its unique ligand CD70 provides costimulation and favors T cell survival (14, 15). The expression of CD70 is tightly regulated
and only transiently present on dendritic cells, B cells, and T cells upon activation, which ensures that signaling through CD27 is limited during times of infection or inflammation (16, 17). CD27 enhances T cell responses through multiple mechanisms that include induction of autocrine IL-2 production and shifting the balance of pro- and antiapoptotic molecules toward a prosurvival state (18–20). The crucial role of CD70-driven costimulation becomes evident postinfection with acute viruses such as vaccinia virus, vesicular stomatitis virus, and the A/NT/60/68 strain of the influenza virus, during which CD70 and CD27 essentially contribute to the size of the CD8 T cell response (14, 21). Recently, we found that CD70 does not have an apparent role in the regulation of the size of primary CD8 T cell responses postinfection with other strains of influenza (22). Close inspection revealed that CD27 favors the outgrowth of low-affinity CD8 T cells at the cost of high-affinity CD8 T cells, suggesting that the costimulatory effects of CD27 depend on the affinity of the Ag interactions (22).

In this study, we addressed whether signaling through CD70 and CD27 directly impairs CD8 T cell differentiation. We found that costimulation through CD70 and CD27 enhances T cell survival after low antigenic stimulation, but increases T cell apoptosis upon high antigenic stimulation. Using in vitro and in vivo settings, we show that CD70-driven costimulation engages the Fas pathway of apoptosis to induce T cell death.

Materials and Methods

Mice

C57BL/6, OTI, lpr, gld (all from The Jackson Laboratory), Cd27+/− (14), Cd27+/− × OTI (22), CD70-B cell Tg (26), Cd27+/− × CD70-B cell Tg (26), CD70-T cell Tg (25), and lpr × CD70-T cell Tg mice (27) were maintained in specific pathogen-free conditions at the animal department of the Academic Medical Center (Amsterdam, The Netherlands). Unless explicitly stated otherwise, CD70-T cell Tg mice that express CD70 on T cells are unable to maintain memory populations of CD8 T cells after influenza infection (25). Taken together, this suggests that under certain conditions, CD70 is able to downregulate CD8 T cell responses, possibly at the level of apoptosis.

Reverse transcriptase multiplex ligation-dependent probe amplification

Total RNA was extracted using TRIzol reagent (Invitrogen); cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Fermentas) and random hexamers (Invitrogen) in combination with poly T oligonucleotides (Invitrogen). Quantitative real-time PCR was performed on a StepOnePlus RT-PCR system (Applied Biosystems). Transcription levels were obtained using FAST SYBR Green Mix (Applied Biosystems). The following primer sets were used for determining hypoxanthine phosphoribosyltransferase (HPRT) and Fasl levels: HPRT (forward: 5’-TGAAGGCTACTCTGAATGATCA- GTCAC-3’ and reverse: 5’-AGCAAGCTCAGCACTTTAACAA-3’) and Fasl (forward: 5’-TACCACCCCGATCAACACC-3’ and reverse: 5’-GAT- TTGGTGTGGGTCTTCTTCTC-3’). Values are represented relative to that of HPRT and calibrated relative to expression in naive CD4+ T cells.

Flow cytometry

Spleen, lungs, and mediastinal lymph nodes (mLN) were isolated and stained with monoclonal antibodies (mAbs) from BD Biosciences or eBioscience. Total polyclonal T cells were isolated from the spleen of wild-type (WT) mice using anti-CD4– and anti-CD8–coated microbeads (Miltenyi Biotec). To measure proliferation, T cells were labeled with 1 µM CFSE (Molecular Probes) in PBS for 10 min at 37°C. As APCs, splenocytes of Cd27+/− mice (referred to as control) and Cd27+/− × CD70-B cell Tg mice (referred to as CD70 Tg) were used. To analyze the effect of endogenous CD70 in these culture assays, we have made use of activated WT B cells as APCs. WT B cells were isolated using anti-CD19 microbeads (Miltenyi Biotec) and stimulated for 4 d in the presence of 5 µg/ml anti-IgM (HB88), 5 µg/ml LPS (Sigma-Aldrich), and 5 µg/ml anti-CD40 (FGK45); kind gift of Louis Boon, Biocerus BV). We have also used mock and CD70-transfected COS cells as APCs in other experiments, as indicated. For the generation of COS-CD70 cells, murine CD70 mRNA was isolated from purified B cells stimulated in vitro for 3 d with LPS and anti-igG and converted into cDNA using Superscript II reverse transcriptase (Invitrogen). Murine CD70 cDNA was amplified using primers (5’-TTGGATCCATGCTGCAAGGAAGGGTCC-3’ and reverse primers (5’-TTGGCGGCGCGCTGAAGGATATCCACTG-3’) and cloned into pcDNA3.1 (Invitrogen). Linearized plasmid was transfected into COS cells with Fugene 6 (Roche), and cells were selected with G418 (Invitrogen). COS-CD70 cells were subjected to multiple rounds of sorting by flow cytometry to select for cells expressing high levels of CD70. APCs were irradiated (30 Gray) and labeled with 1 µM DDAO (Molecular Probes) in PBS for 10 min at 37°C. Cells and APCs were cocultured in a 1:1 ratio in 96-well, round-bottom plates (Corning) containing 200 µl RPMI 1640 10% FCS. OTI cells were stimulated with the indicated concentration of the cognate peptide SIINFEKL, and polyclonal T cells were stimulated with anti-CD3 (145-2C11; kind gift of Louis Boon, Biocerus BV) that was coated onto the 96-well round-bottom plates for 1 h in PBS with 0.5% BSA. Blood, spleen, and lung cell suspensions were subjected to erythrocyte lysis (155 mM NH4Cl, 10 mM KHCO3, and 1 mM EDTA) to remove RBCs. An automated cell counter (Counter, Innovatis, or Beckman Coulter Counter) was used to obtain absolute cell numbers. Cell suspensions were stained with fluorescein-conjugated or biotinylated primary Abs for 30 min at 4°C in PBS 0.5% BSA. For biotinylated Abs, fluorescein-conjugated streptavidin (eBioscience) was used as secondary antibodies. PLL/IRI blockers were used for nonspecific binding of Abs (2.4G2; kind gift of Louis Boon, Biocerus BV).

Quantitative PCR

RNA was extracted using TRIZol reagent (Invitrogen); cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Fermentas) and random hexamers (Invitrogen) in combination with poly T oligonucleotides (Invitrogen). Quantitative real-time PCR was performed on a StepOnePlus RT-PCR system (Applied Biosystems). Transcription levels were obtained using FAST SYBR Green Mix (Applied Biosystems). The following primer sets were used for determining hypoxanthine phosphoribosyltransferase (HPRT) and Fasl levels: HPRT (forward: 5’-TGAAGGCTACTCTGAATGATCAGTCAC-3’ and reverse: 5’-AGCAAGCTCAGCACTTTAACAA-3’) and Fasl (forward: 5’-TACCACCCCGATCAACACC-3’ and reverse: 5’-GATTGGTGTGGGTCTTCTTCTC-3’). Values are represented relative to that of HPRT and calibrated relative to expression in naive CD4+ T cells.

Western blot

Cells were lysed in 1% Triton X-100–containing lysis buffer at the indicated time points after stimulation, and cell lysates were boiled in sample buffer for Western blot analysis. SDS-PAGE gel electrophoresis was performed using the Bio-Rad mini-PROTEAN electrophoresis system (Bio-Rad). Lysates were analyzed using primary Abs against Bim (Stressgen), Bel-ⅩI (Transduction Laboratories), and β-actin (Santa Cruz Biotechnology) and secondary IRDye 680- or 800-labeled Abs (Li-Cor). Western blots were visualized on an Odyssey Imager (Li-Cor). Densitometric analysis was performed using Odyssey Application software v3.0 (Li-Cor).

In vitro T cell stimulation

OTI cells were isolated from the spleen of OTI TCR Tg mice using anti-CD8–coated beads, and total polyclonal T cells were isolated from the spleen of wild-type (WT) mice using anti-CD4– and anti-CD8–coated microbeads (Miltenyi Biotec). To measure proliferation, T cells were labeled with 1 µM CFSE (Molecular Probes) in PBS for 10 min at 37°C. As APCs, splenocytes of Cd27+/− mice (referred to as control) and Cd27+/− × CD70-B cell Tg mice (referred to as CD70 Tg) were used. To analyze the effect of endogenous CD70 in these culture assays, we have made use of activated WT B cells as APCs. WT B cells were isolated using anti-CD19 microbeads (Miltenyi Biotec) and stimulated for 4 d in the presence of 5 µg/ml anti-IgM (HB88), 5 µg/ml LPS (Sigma-Aldrich), and 5 µg/ml anti-CD40 (FGK45; kind gift of Louis Boon, Biocerus BV). We have also used mock and CD70-transfected COS cells as APCs in other experiments, as indicated. For the generation of COS-CD70 cells, murine CD70 mRNA was isolated from purified B cells stimulated in vitro for 3 d with LPS and anti-igG and converted into cDNA using Superscript II reverse transcriptase (Invitrogen). Murine CD70 cDNA was amplified using primers (5’-TTGGATCCATGCTGCAAGGAAGGGTCC-3’ and reverse primers (5’-TTGGCGGCGCGCTGAAGGATATCCACTG-3’) and cloned into pcDNA3.1 (Invitrogen). Linearized plasmid was transfected into COS cells with Fugene 6 (Roche), and cells were selected with G418 (Invitrogen). COS-CD70 cells were subjected to multiple rounds of sorting by flow cytometry to select for cells expressing high levels of CD70. APCs were irradiated (30 Gray) and labeled with 1 µM DDAO (Molecular Probes) in PBS for 10 min at 37°C. Cells and APCs were cocultured in a 1:1 ratio in 96-well, round-bottom plates (Corning) containing 200 µl RPMI 1640 10% FCS. OTI cells were stimulated with the indicated concentration of the cognate peptide SIINFEKL, and polyclonal T cells were stimulated with anti-CD3 (145-2C11; kind gift of Louis Boon, Biocerus BV) that was coated onto the 96-well round-bottom plates for 1 h in PBS with 0.5% BSA. Blood, spleen, and lung cell suspensions were subjected to erythrocyte lysis (155 mM NH4Cl, 10 mM KHCO3, and 1 mM EDTA) to remove RBCs. An automated cell counter (Counter, Innovatis, or Beckman Coulter Counter) was used to obtain absolute cell numbers. Cell suspensions were stained with fluorescein-conjugated or biotinylated primary Abs for 30 min at 4°C in PBS 0.5% BSA. For biotinylated Abs, fluorescein-conjugated streptavidin (eBioscience) was used as secondary antibodies. PLL/IRI blockers were used for nonspecific binding of Abs (2.4G2; kind gift of Louis Boon, Biocerus BV). Flow cytometry was performed using FACS Calibur and Canto flow cytometers (BD Biosciences).
Influenza infection and adoptive transfer

Mice were intranasally infected with \(10^3\) TCID50 of the H1N1 influenza A virus A/PR8/34 for analysis of primary immune responses. Heterotypic infection with \(10^3\) TCID50 of the H3N2 influenza A virus HKx31 and \(10^3\) TCID50 A/PR8/34 was performed to examine secondary responses. Blood samples were drawn from the tail vein to determine the levels of influenza-specific CD8 T cells at the specified days postinfection. Mice were sacrificed, and blood, spleen, mLN, and lungs were collected for analysis at the indicated days postinfection. Influenza-specific CD8 T cells were enumerated using anti-CD8 Abs and PE- or allophycocyanin-conjugated tetramers of H-2Db containing the influenza-derived peptide NP366–374 ASNENMETM (Sanquin Reagents). For adoptive transfer, CD8 T cells were purified from the spleen by selection using anti-CD8–coated microbeads (Miltenyi Biotec), and \(10^3\) CD8 T cells were i.v. injected per recipient mouse.

LCMV infection

Mice were i.v. infected with \(2 \times 10^6\) PFU LCMV clone 13 virus for analysis of chronic immune responses. Mice were sacrificed, and spleens were collected for analysis at the indicated days postinfection. LCMV-specific CD8 T cells were enumerated using allophycocyanin-conjugated tetramers of H-2D\(b\) containing the LCMV-derived peptide GP33–41 KA VYNFATC (Sanquin Reagents).

Statistical analysis

All experiments have been performed at least twice with similar results. Figures represent means, and error bars denote SEM. Two-tailed Student \(t\) test was used to analyze for statistical significance. \(p\) value, \(0.05\) was considered statistically significant (* \(p < 0.05\), ** \(p < 0.005\)).

Results

The outcome of CD70-driven costimulation depends on antigenic strength

Upon binding of CD70, CD27 enhances expansion of T cells by improving survival (15) and proliferation (28). Indeed, as previously published (18, 28), we found that TCR Tg OVA-specific OTI cells activated with low amounts of peptide proliferate faster and/or display enhanced survival in the presence of CD70 Tg APCs compared with control APCs (Fig. 1A). Cd27\(^{-/-}\) OTI cells
did not have this advantage, showing that interactions between CD70 and CD27 are required for proliferation and/or survival of OTI cells to occur after low antigenic triggering (Fig. 1A). Using PI to examine T cell death, we observed that proliferating WT OTI cells remained alive in contrast to Cd27−/− OTI cells after stimulation with low doses of peptide in the presence of CD70 Tg APCs (Fig. 1A, 1B). Strikingly, under higher concentrations of peptide, the positive effects of CD70 and CD27-driven costimulation on T cell proliferation and/or survival were not apparent (Fig. 1A, 1B). In fact, activation of WT OTI cells, but not Cd27−/− OTI cells, with high amounts of peptide resulted in enhanced cell death in the presence of CD70 Tg APCs compared with control APCs (Fig. 1B). Staining for Annexin V demonstrated that CD70-driven T cell death under these conditions occurred through apoptosis (Fig. 1C). Next, we tested whether endogenous CD70 was able to induce similar effects in OTI cells as Tg CD70. Therefore, isolated WT B cells were stimulated with anti-IgM, LPS, and anti-CD40, which has been shown previously to induce endogenous CD70 expression (29). Although the expression level of CD70 on activated WT B cells was lower than on B cells of CD70 Tg mice (Fig. 1D), these levels of endogenous CD70 expression were sufficient to enhance proliferation of OTI cells after stimulation with low doses of peptide and to induce cell death of OTI cells after stimulation with high doses of peptide (Fig. 1E, 1F). Taken together, this shows that CD70 induces T cell death rather than T cell proliferation after strong antigenic triggering.

Cell-intrinsic and -extrinsic factors mediate CD70-driven costimulation

To study whether soluble or cell-bound factors mediated the costimulatory effects of CD70 and CD27, we set up a coculture system, in which Ly5.1+/− WT and Ly5.2+/− Cd27−/− OTI cells were mixed in a 1:1 ratio and stimulated with peptide in the presence of CD70 Tg APCs. After stimulation with low doses of peptide, Cd27−/− OTI cells cocultured with WT OTI cells proliferated more extensively than Cd27−/− OTI cells cultured separately on CD70 Tg APCs (Fig. 2A). This shows that the Cd27−/− OTI cells benefited from CD70-driven costimulation of WT OTI cells. Despite this benefit on proliferation, Cd27−/− OTI cells displayed a higher degree of cell death compared with cocultured WT OTI cells on CD70 Tg APCs (Fig. 2B). Thus, under low antigenic triggering, CD70 induced costimulation through a cell-extrinsic factor that increased T cell proliferation and through a cell-intrinsic factor that enhanced T cell survival. In the presence of intermediate Ag doses, the beneficial effects of CD70-driven costimulation disappeared, and proliferation and cell death of WT and Cd27−/− OTI cells in the presence of CD70 Tg APCs was similar under separate and coculture conditions (Fig. 2A, 2B). Strikingly, the enhanced cell death that was observed after stimulation of WT OTI cells with high amounts of peptide in the presence of CD70 Tg APCs was also evident in cocultured Cd27−/− OTI cells, but not in separately cultured Cd27−/− OTI cells (Fig. 2B). This indicates that CD70-driven T cell death after strong antigenic triggering is mediated by the extrinsic pathway rather than the intrinsic pathway of apoptosis.

CD70 induces T cell death through FasL and Fas

Fas (CD95) is an important death-inducing receptor that mediates activation-induced cell death of T cells upon binding to its ligand FasL (CD178). To examine whether CD27-driven costimulation activated FasL and Fas-driven apoptosis, we analyzed expression of FasL and Fas on OTI cells in response to peptide stimulation and CD27 triggering. CD70 induced expression of Fas on OTI cells, but the contribution was not very prominent in the presence of the higher doses of peptide Ag that were required to induce CD70-driven T cell death (Fig. 3A). FasL was also upregulated on OTI cells in the presence of CD70-driven costimulation, but in contrast to the induction of Fas, this effect occurred in particular after stimulation with intermediate to high peptide doses (Fig. 3A). Consistent with the induction of FasL protein, CD70 induced expression of FasL at the mRNA level in the presence of high but not low doses of peptide (Fig. 3B). This suggested that CD70 induced T cell death predominantly through transcriptional up-regulation of FasL expression and engagement of the Fas pathway of apoptosis. Indeed, blockade of FasL–Fas signaling through blocking anti-FasL Abs impaired CD70-induced cell death in WT OTI cells (Fig. 3C), but did not have a dramatic effect on cell death of Cd27−/− OTI cells (Fig. 3D). Thus, strong antigenic stimulation allows CD70 to induce T cell death via upregulation of FasL and subsequent triggering of Fas.

To examine whether costimulation through CD70 and CD27 similarly affected polyclonal T cell populations after high antigenic triggering, total T cells of naive WT mice were stimulated with high levels of anti-CD3 Abs in the presence of control or CD70 Tg APCs. We did not observe that CD70 augmented the survival or the proliferative capacity of CD4 and CD8 T cells under these conditions (Fig. 4A and data not shown). In contrast, the presence of CD70 Tg APCs increased T cell death in both CD4 and CD8 T cells (Fig. 4A, 4B). T cells of Cd27−/− mice were not affected by CD70-driven costimulation (Fig. 4A, 4B). Staining for Annexin V demonstrated that CD70 enhanced cell death of WT but not Cd27−/− T cells through apoptosis (Fig. 4C). To examine whether these findings were reproducible using different APCs, we made use of COS cells that were transfected with CD70. In contrast to mock-transfected COS cells, CD70-transfected COS

![Figure 2](http://www.jimmunol.org)
CD70 induces Fas-driven T cell death despite costimulatory activity

Costimulation through CD70 and CD27 has also been shown to induce upregulation of IL-2 and the antiapoptotic Bcl-2 family member Bcl-XL, which promotes T cell survival (18–20). We questioned whether CD70 differentially regulated expression of IL-2 and Bcl-2 family members in T cells after stimulation with high antigenic doses. The production of IL-2 was analyzed using ELISA in OTI cells stimulated with increasing amounts of peptide in the presence or absence of CD70-driven costimulation. After triggering of CD70, WT but not Cd27−/− OTI cells produced much higher amounts of IL-2, particularly in the presence of high doses of Ag (Fig. 5A). Similarly, CD70 induced production of IL-2 in polyclonal T cells stimulated with high amounts of anti-CD3 (Fig. 5B). The expression of Bcl-2 family members was determined using reverse-transcriptase multiplex ligation-dependent probe amplification in strongly activated T cells of WT mice in the presence or absence of CD70-driven costimulation. Under conditions that result in enhanced T cell death, costimulation through CD70 and CD27 induced upregulation of Bcl-XL and Bcl-2 expression. Interestingly, CD70 also induced downregulation of Bim expression, but did not interfere with expression of other molecules involved in apoptosis or survival of T cells (Fig. 5C). To confirm these findings at the protein level, Bcl-XL and Bim expression were analyzed by Western blot in T cells after stimulation with mock or CD70-transfected COS cells. CD70 upregulated the expression of Bcl-XL (Fig. 5D) and downregulated the expression of Bim in T cells after 24–48 h of stimulation (Fig. 5E). The effect of CD70 was in particular strong on the largest isoform of Bim (Bim EL) and occurred to a lesser extent on the other isoforms of Bim (Fig. 5E). Thus, under these conditions, CD70 and CD27 shifted the balance of Bcl-2 molecules in T cells toward an apoptosis-resistant state. This supports the notion that CD70 elicits costimulatory activity after stimulation of T cells with high antigenic loads despite the induction of T cell apoptosis.

CD70 accelerates accumulation of Fas-sensitive B220+ double-negative T cells in vivo

To study the Fas-dependent effects of CD70 and CD27 signaling in vivo, mice that constitutively express CD70 on T cells (25) were crossed onto Fas-deficient lpr mice. We observed that these mice had dramatically enlarged LNs compared with WT, CD70 Tg, and lpr mice (Fig. 6A). In particular, cervical and axillary LNs were enlarged (Fig. 6A). The enlarged LNs of lpr × CD70 Tg mice correlated with the unusual presence of a large proportion of double-negative (DN) T cells that, in contrast to conventional CD4 and CD8 T cells, expressed the B cell marker B220 (Fig. 6B). B220+ DN T cells are normally eliminated within lymphoid tissue through Fas-dependent apoptosis and therefore are hardly detectable within WT mice, but accumulate in lpr mice (30). Similarly, patients with mutations in FasL or Fas develop autoimmune lymphoproliferative syndrome that is manifested by a dramatic increase in DN T cells and consequent enlargement of, in particular, the cervical LNs (31). We observed that the accumulation of B220+ DN T cells was much larger within LNs and spleen of lpr × CD70 Tg mice than in those of lpr mice (Fig. 6C). Interestingly, lpr × Bim−/− mice displayed a similar phenotype as lpr ×
CD70 Tg mice, as they also had strongly increased B220+ DN T cell numbers (11–13). The dramatic increase of B220+ DN T cells in lpr3 CD70 Tg mice is consistent with a role for CD70 in the downregulation of Bim (see also Fig. 5C, 5E) that, in the absence of Fas, enables enhanced survival of B220+ DN T cells. Moreover, this may imply that CD70-driven inflammation may accelerate the onset of autoimmune lymphoproliferative syndrome in patients with mutations in Fas or FasL.

CD70-driven costimulation triggers Fas-dependent T cell death in vivo

We took advantage of the lpr × CD70 Tg mice to study whether CD70 impacted differentiation of conventional T cells in a Fas-dependent manner. CD70 Tg mice that express CD70 on T cells, B cells, or dendritic cells have increased numbers of effector phenotype T cells compared with WT mice, underlining the role of CD70 in costimulation of T cells (25, 26, 32). Indeed, staining for CD44 and CD62L in the CD8 T cell compartment of the spleen showed enhanced percentages and absolute numbers of CD44hiCD62Llo effector or effector memory phenotype CD8 T cells in T-cell specific CD70 Tg mice compared with WT mice (Fig. 7A, 7B). The CD44hiCD62Llo CD8 T cell population in CD70 Tg mice was predominantly of the effector phenotype, as the majority lacked expression of the memory-associated molecule CD127 (Fig. 7A, 7B). Strikingly, in the absence of Fas, CD70 induced the accumulation of not only effector CD8 T cells, but also of effector memory CD8 T cells (Fig. 7A, 7C). This demonstrates that CD70-driven expansion of effector phenotype and, in particular, of effector memory phenotype T cells is limited by CD70-induced and Fas-dependent T cell death.
CD70 signaling during the primary response downregulates CD8 T cell recall responses

To study the timing of CD70-induced elimination of T cells during in vivo immune responses, we used the influenza infection model. Previously, we have described that the constitutive presence of CD70-dependent costimulation enhances primary CD8 T cell responses against influenza (25, 33). In contrast, secondary CD8 T cell responses are impaired under chronic CD70-driven costimulation through the loss of memory CD8 T cells (25). We were interested whether CD70 signaling during the primary response or, thereafter, during the memory phase was important for the loss of memory CD8 T cells and impaired recall CD8 T cell responses. Therefore, WT and CD70 Tg mice were infected with influenza, and total CD8 T cells were isolated at day 10 postinfection for transfer into noninfected WT mice. To prevent that adoptively transferred T cells expressed CD70, we made use of CD70 Tg mice that express CD70 on B cells. These CD70 Tg mice have a similar phenotype as CD70 Tg mice that express CD70 on T cells (25, 26).Recipient mice were infected with influenza 45 d after transfer of donor CD8 T cells, and the number of influenza-specific CD8 T cells of donor origin was analyzed 11 d later (Fig. 8A). Donor CD8 T cells of WT mice, but not of CD70 Tg mice, contributed significantly to the influenza-specific CD8 T cell response in recipient WT mice (Fig. 8B). Quantification of donor-derived CD8 T cells showed that those of WT origin, in contrast to those of CD70 Tg origin, were able to induce strong recall responses in blood, mLN, spleen, or lungs upon infection (Fig. 8C, 8D). This implied that expression during the primary infection is sufficient for CD70 to induce impairment of secondary CD8 T cell responses. To establish whether expression of CD70 during the memory phase also contributed to impaired recall CD8 T cell responses, the reciprocal experiment was performed, in which CD8 T cells of influenza-infected WT mice were transferred into WT or CD70 Tg recipients (Fig. 8E). Donor CD8 T cells of WT mice contributed significantly to the influenza-specific CD8 T cell response of WT and CD70 Tg recipients (Fig. 8F). Strong recall responses of WT donor CD8 T cells were observed in mLN, spleen, and lungs upon infection of both WT
CD70 enables Fas-dependent elimination of CD8 T cells

Recently, it has been reported that CD70-driven costimulation reduces rather than enhances CD8 T cell responses during chronic infection with LCMV clone 13 (24). Consistent with this study, we observed that Cd27<sup>-/-</sup> mice displayed enhanced CD8 T cell responses against LCMV clone 13 compared with WT mice (Fig. 9A). Interestingly, Cd27<sup>-/-</sup> mice already had enlarged CD8 T cell responses at day 8 postinfection, and the numbers of LCMV-specific CD8 T cells remained elevated until at least day 14 postinfection (Fig. 9B). Thus, CD70 instructs downregulation of memory CD8 T cell responses early during primary infection when Ag levels are high and not during the memory phase when the virus has been cleared.

Not only higher in the blood, but also in the spleen of lpr × CD70 Tg mice compared with CD70 Tg mice (Fig. 9D). This shows that CD70 is able to trigger Fas-dependent elimination of CD8 T cells. The impaired removal of CD8 T cell responses in lpr × CD70 Tg mice was not caused by reduced viral clearance, as the influenza virus was undetectable in the lungs of all mice (data not shown). This is corroborated by earlier observations that show that Fas is not essential for elimination of virally infected cells after influenza infection (34). Reduced removal of effector CD8 T cells resulted in larger memory CD8 T cell responses in lpr × CD70 Tg mice compared with CD70 Tg mice (Fig. 9C, 9E). Levels of memory CD8 T cells in blood and spleen of lpr × CD70 Tg mice were almost restored to WT and lpr levels (Fig. 9E). Thus, CD70-driven costimulation enables Fas-dependent elimination of influenza-specific CD8 T cells in the contraction phase, which results in the maintenance of lower numbers of memory CD8 T cells.

Discussion

In this study, we have demonstrated that Fas-driven apoptosis forms an integral aspect of CD70-driven costimulation that suppresses the prosurvival effects of CD27 on T cells. This striking role of costimulatory CD70 and CD27 in downregulation of CD8 T cell responses may clarify the negative impact of CD27 signaling during previously reported in vitro and in vivo settings.

Members of the TNFR family, including CD27, mediate an important pathway of costimulation that essentially contributes to expansion of CD8 T cells during acute immune responses (35). CD27 induces expansion of CD8 T cells through enhancement of proliferation and survival (15, 28). We also observed that CD70 enhances the proliferation and survival of CD8 T cells in our
in vitro cultures after stimulation with low doses of antigenic peptide. The effect on T cell proliferation is separable from the prosurvival effect of CD27, underlining the dual ability of CD27 to enhance expansion of CD8 T cells through proliferation and survival. A multitude of complementary mechanisms accounts for the costimulatory activity of CD27, including induction of IL-2 production and upregulation of antiapoptotic members of the Bcl-2 family (18–20). Confirming these studies, we have shown that CD70 induced production of IL-2 and upregulated antiapoptotic Bcl-XL. Moreover, we observed that CD70 induced downregulation of proapoptotic Bim, indicating that Bim-dependent T cell apoptosis is impaired during CD70-driven costimulation.

In contrast to these prosurvival effects of CD27, we found that CD27 is also able to induce T cell death through induction of FasL and Fas-dependent apoptosis after stimulation with high amounts of Ag. Previously, we have described that CD70 sensitizes T cells for Fas-driven apoptosis, as agonistic anti-Fas Abs were more effective in triggering T cell apoptosis in the presence of CD70.

**FIGURE 8.** CD70 costimulation in the primary response reduces recall CD8 T cell responses. (A–D) WT and CD70-B cell Tg mice were infected with the influenza strain HKx31, and after 10 d, total CD8 T cells were isolated, and $10^7$ cells were transferred into Ly5.1$^+$ WT recipients. After 45 d, recipient mice were infected with the influenza strain A/PR8/34, and 11 d later, they were sacrificed for analysis. (A) Schematic representation is shown of the experimental setup to determine whether CD70 eliminates memory CD8 T cells during the priming phase of influenza infection. (B) Representative dot plots display influenza-specific CD8 T cells by CD8 and tetramer staining of splenocytes at day 11 of rechallenge (top panel). Tetramer$^+$ CD8 T cells were gated to display host- and donor-derived cells by Ly5.1 staining (bottom panel). (C) The percentage of tetramer$^+$ donor CD8 T cells (Ly5.1$^+$) postinfection was followed in time within the blood. (D) The percentage of tetramer$^+$ donor CD8 T cells (Ly5.1$^+$) was determined within the CD8 T cell population of mLN, spleen, and lungs at day 11 after rechallenge. (E–G) Ly5.1$^+$ WT mice were infected with HKx31, and after 17 d, total CD8 T cells were isolated and transferred into WT or CD70 Tg recipients. After 52 d, recipient mice were infected with A/PR8/34 45, and 10 d later, they were sacrificed for analysis. (E) Schematic representation is shown of the experimental setup to determine whether CD70 eliminates memory CD8 T cells during the memory phase of influenza infection. (F) Representative dot plots of total splenocytes display staining for CD8 and tetramer to enumerate influenza-specific CD8 T cells at day 10 after rechallenge (top panel). Tetramer$^+$ CD8 T cells were gated to display host- and donor-derived cells using Ly5.1 staining (bottom panel). (G) The percentage of tetramer$^+$ donor CD8 T cells (Ly5.1$^+$) was determined within the CD8 T cell population of mLN, spleen, and lungs at day 10 after rechallenge. **p < 0.005.
tetramer+ CD8 T cells was followed in time within the blood of CD70 Tg animals upon infection with influenza. (D) The absolute number of tetramer+ CD8 T cells was analyzed in mLN, spleen, and lungs of CD70 Tg and lpr × CD70 Tg mice at day 64 after influenza infection. (E) The absolute number of tetramer+ CD8 Tg cells was analyzed in spleen, mLN, and lungs of WT and lpr mice at day 59 after influenza infection (left panel) and CD70 Tg and lpr × CD70 Tg mice at day 64 after influenza infection (right panel). *p < 0.05, **p < 0.005.

FIGURE 9. CD70 and Fas eliminate CD8 T cells during infection. (A) WT and Cd27−/− mice were infected with LCMV clone 13, and the number of LCMV-specific CD8 T cells was enumerated in spleen after 8 and 20 d of infection using H2-D^b tetramers loaded with GPe33–41 peptide. (B) Similarly, the number of LCMV-specific CD8 T cells was quantified in spleen of WT and lpr mice at day 8 postinfection. (C) The percentage of tetramer+ CD8 T cells was followed in time within the blood of CD70 Tg and lpr × CD70 Tg animals upon infection with influenza. (D) The absolute number of tetramer+ CD8 T cells was analyzed in mLN, spleen, and lungs of CD70 Tg and lpr × CD70 Tg mice at day 14 after influenza infection. (E) The absolute number of tetramer+ CD8 Tg cells was analyzed in spleen, mLN, and lungs of WT and lpr mice at day 59 after influenza infection (left panel) and CD70 Tg and lpr × CD70 Tg mice at day 64 after influenza infection (right panel). *p < 0.05, **p < 0.005.

(36). Our current findings show that CD70 instructs rather than permits Fas-driven apoptosis, as CD70 upregulated the expression of FasL on activated T cells and engaged Fas-dependent cell death without requirement for external stimulation of the Fas pathway. CD70 and CD27 triggered T cell death despite the induction of IL-2 production and the stimulatory and suppressive effects on expression of Bcl-Xl and Bim, respectively. Previously, it has been documented that activation-induced cell death, which involves repetitive exposure of T cells to Ag occurs in an IL-2–dependent manner (37). Therefore, it is possible that the induction of IL-2 does not conflict with T cell apoptosis during CD70-driven costimulation. Consistent with a supportive role for IL-2 in CD70-induced T cell apoptosis, IL-2 triggers activation-induced cell death through downregulation of the expression of cFLIP that antagonizes Fas-driven apoptosis and upregulation of FasL expression (38). Moreover, it has been reported previously that CD70-driven expansion of T cells is more pronounced in the absence of IL-2 (28). In T cells, Fas triggers apoptosis independent of Bcl-XI and Bim, which enables CD27 signaling to induce T cell death during suppression of the intrinsic pathway of apoptosis (39). Thus, Fas-dependent apoptosis may represent a negative-feedback mechanism of CD27 signaling that enables CD27 to counteract its effects on CD8 T cell expansion.

We show that CD70-driven Fas-dependent apoptosis occurs after stimulation with higher levels of Ag than the prosurvival effects of CD70. Therefore, the opposite effects of CD70 may optimize the size of CD8 T cell responses. Under conditions of limiting Ag, CD70 may promote expansion of effector CD8 T cells, whereas during Ag abundance when survival factors for CD8 T cells are optimal, CD70 may enable removal of CD8 T cells. Consistent with this, during acute infection such as with influenza, when viral and antigenic loads are likely limiting, CD70 enhances the survival of effector and memory CD8 T cells (14, 15). Interestingly, it has been reported that the prosurvival effects of CD70 on CD8 T cell responses against influenza depend at least in part on the capacity of CD70 to prevent FasL upregulation on CD4 T cells and consequent Fas-dependent elimination of CD8 T cells (40). A role for CD70 in downregulation of CD8 T cell responses may have relevance for chronic infection, during which Ag and Ag-related survival signals persist, and continuous turnover of effector CD8 T cells occurs to ensure the replacement of senescent or ineffective T cells. This is consistent with the increased numbers of effector CD8 T cells under blockade of CD70-driven costimulation that we and others observed during infection with chronic strains of the LCMV virus (23, 24). The role of Fas-driven apoptosis is also more apparent during chronic infection than during acute infection. Fas and Bim cooperate to limit CD8 T cell numbers in the chronic model of murine γ-herpes virus infection in contrast to the acute model of HSV infection, during which contraction is exclusively Bim dependent (11). Extending these studies, we have found that Fas also eliminates effector CD8 T cells during chronic infection with LCMV clone 13. Taken together, these studies highlight the importance of CD70 and Fas in restricting CD8 T cell responses during infection, and in this study, we clarify that they in fact operate in the same pathway.

We have previously shown that phenotypically, CD70 Tg mice that display constitutive expression of CD70 on B or T cells display remarkable similarities to situations of chronic infection (25, 27, 41). Constitutive expression of CD70 on B cells induces effector CD8 T cell differentiation that eventually culminates in depletion of the naive T cell pool and lethal immunodeficiency (41). The accumulation of effector CD8 T cells and lethal disease was dramatically accelerated in B cell-specific CD70 Tg mice on a Fas-deficient background (36). This has shown the importance of Fas in restricting the pathological impact of constitutive CD70 expression, but the rapid onset of disease and death has prevented careful analysis of the events during CD8 T cell differentiation. In this study, using T cell-specific lpr × CD70 Tg mice that display a similar but milder phenotype than B-cell specific lpr × CD70 Tg mice, we have demonstrated that memory rather than effector phenotype CD8 T cells are targeted by CD70-induced and Fas-dependent apoptosis. The development of memory CD8 T cells that are able to provide enhanced protection upon reinfection is a cardinal feature of acute CD8 T cell responses. However, differentiation into memory CD8 T cells is compromised after chronic LCMV infection. LCMV-specific CD8 T cells that are transferred from chronically infected mice into noninfected mice do not survive in the absence of Ag (42). Supporting a role of CD70 in this process, we have previously observed the disappearance of bona fide memory CD8 T cells after acute infection with influenza in the presence of CD70 (25). In this study, we have demonstrated that constitutive expression of CD70 during the memory phase did not drive the loss of these memory CD8 T cells. Rather, expression of CD70 during the primary response compromised CD8 T cell memory in a Fas-dependent fashion. This
elminates long-term signaling through CD70 and CD27 as the main driving force of downregulation of CD8 T cell responses in this setting. The early effect of CD70 on elimination of CD8 T cell memory was consistent with the timeframe, in which CD70 induced Fas-driven removal of CD8 T cells during influenza infection. This indicates that CD70 and CD27 mediate a Fas-dependent pathway that compromises the differentiation of memory CD8 T cells during chronic infection.

The dual capacity of CD27 to up- and downregulate T cell responses is shared by the related TNFR-like molecule 4-1BB. During murine CMV infection, 4-1BB suppresses CD8 T cell responses against acute epitopes, but enhances CD8 T cell responses during the latent phase (43). Supporting this study, agonistic anti-4-1BB Abs improve or impair T cell responses against acute infection with LCMV and influenza depending on the time of application (44). Similar to CD27, 4-1BB required Fas-driven apoptosis to induce contraction of CD8 T cell responses (44). The costimulatory pathway through CD28 has not been reported to downregulate T cell responses by itself but is counteracted by the inhibitory family member CTLA4 that also binds B7 molecules (45). This shows that the ability to adequately control T cell responses through negative feedback is an important and inherent aspect of costimulation. Therefore, it is important to take the potential to downsize immune responses into account in the design of vaccination strategies that target costimulation either through CD70 and CD27 or other pathways.

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Disclosures

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References


