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Stimulation of the costimulatory receptor CD27 by its ligand CD70 has proved important for the generation of primary and memory CD8+ T cell responses in various models of antigenic challenge. CD27/CD70-mediated costimulation promotes the survival of primed T cells and thereby increases the size of effector and memory populations. In this paper, we reveal molecular mechanisms underlying the prosurvival effect of CD27. CD27 signaling upregulated expression of the antiapoptotic Bcl-2 family member Bcl-xL. However, genetic reconstitution of Cd27−/− CD8+ T cells with Bcl-xL alone or in combination with the related protein Mcl-1 did not compensate for CD27 deficiency in the response to influenza virus infection. This suggested that CD27 supports generation of the CD8+ effector T cell pool not only by counteracting apoptosis via Bcl-2 family members. Genome-wide mRNA expression profiling indicated that CD27 directs expression of the Pim1 gene. Pim-1 is a serine/threonine kinase that sustains survival of rapidly proliferating cells by antiapoptotic and prometabolic effects that are independent of the mammalian target of rapamycin (mTOR) pathway. In TCR-primed CD8+ T cells, CD27 could increment Pim-1 protein expression and promote cell survival throughout clonal expansion independent of the mTOR and IL-2R pathways. In addition, introduction of the Pim1 gene in Cd27−/− CD8+ T cells partially corrected their defect in clonal expansion and formation of an effector pool. We conclude that CD27 may contribute to the survival of primed CD8+ T cells by the upregulation of antiapoptotic Bcl-2 family members but also calls the Pim-1 kinase survival pathway into action. The Journal of Immunology, 2010, 185: 000–000.

Using a mouse model of intranasal influenza virus infection, we have shown that the contributions of CD28 and CD27 to the CD8+ T cell response are complementary (6). At the priming site, CD28 and CD27 contributed in equal measure to clonal expansion but by different mechanisms. CD28 promoted cell cycle entry and progression, whereas CD27 had no effect on cell division but promoted CD8+ T cell survival throughout successive cell cycles. The concept that CD28 and CD27 are both required for the CD8+ T cell response was confirmed in models of protein immunization (7) and organ transplantation (8). In the influenza virus model, CD27, 4-1BB, and OX40 all affected the size of same Ag-specific CD8+ effector and/or memory pools, most likely by their sequential action throughout the priming, effector, and contraction phases of the T cell response (9).

Collective data indicate that CD27 and its relatives support the generation of CD8+ effector and memory T cells to a large extent by prosurvival signaling (1). However, the downstream molecular pathways by which this is accomplished have not been fully elucidated. Throughout their existence, T cells are kept alive by various environmental signals (10). When they are naive or in the memory state, the TCR and homeostatic cytokines provide survival input. When T cells are challenged by Ag, costimulatory receptors provide survival signals, with support from cytokines (11, 12). Life/death decisions in lymphocytes primarily proceed via the mitochondrially mediated pathway for caspase activation (12, 13). This pathway is controlled by Bcl-2 family members, with inhibitory Bcl-2 family members blocking apoptotic cell death and the concerted action of BH3-only and Bax/Bak family members promoting death. Rapidly dividing T cells experience stresses of various kinds, which are translated into an apoptotic response via BH3-only proteins (13). Metabolic stress such as glucose deprivation is sensed by Bad and Noxa (14, 15). Cytokine deprivation or DNA damage are sensed by Puma and/or Noxa (16). In the contraction phase, which is characterized by withdrawal of cytokines and costimulatory ligands, BH3-only protein Bim plays a key role (17, 18). BH3-only and Bax/Bak proteins interact with inhibitory Bcl-2 family members in a...
selective manner. In particular, Bcl-xL can bind Bim, Bad, and Puma, but not Noxa, which is only bound by Mcl-1 and Bfl-1. Also, Bcl-xL can interact with both Bax and Bak, whereas Bcl-2 only binds to Bax (19). It follows from this selectivity that complete inhibition of the mitochondrial apoptosis pathway may require combined overexpression of inhibitory Bcl-2 family members that can saturate all BH3-only and Bax/Bak proteins.

A link between TNFR family members and Bcl-2 proteins has been established. In T cells, OX40 upregulates Bcl-2 and Bcl-xL (20), 4-1BB upregulates Bcl-xL and Bfl-1 and downregulates Bim (21–23), and CD27 upregulates Bcl-xL in human CD4+ T cells (24). CD27 and its relatives do not activate tyrosine kinase pathways as do cytokine receptors and CD28. Instead, they bind TNFR-associated factors and signal to NF-κB (1), which counteracts apoptosis via the transcriptional induction of inhibitory Bcl-2 family member Bfl-1 and other antiapoptotic proteins (25).

Two other potent survival signaling pathways are those initiated by the PKB/Akt and Pim serine/threonine kinases, which are primarily implicated downstream from cytokine receptors (26). These pathways are critical for rapidly dividing cells because they inhibit apoptosis and at the same time stimulate cell metabolism and protein synthesis. Both Akt and Pim target Bcl-2 family members. They phosphorylate and inhibit Bad, and the Akt pathway also induces Bcl-xL and inhibits Bim expression (26, 27). Moreover, the Akt pathway increases glucose uptake and induces the transition from oxidative phosphorylation to glycolysis (28). It can also activate mammalian target of rapamycin (mTOR), which regulates protein synthesis by coupling amino acid uptake with increased translation rate (26, 29). Pim1 was originally defined as an oncogene that promotes T cell lymphomagenesis (30), and it is part of a family with three members that act in a partially redundant fashion (31). The Pim kinases are constitutively active and are primarily regulated at the level of gene transcription and protein turnover (32). The exact mechanism of action of Pim kinases remained elusive until it was demonstrated that they counteract mitochondrial apoptosis as well as stimulate metabolism and protein synthesis, independent of the mTOR and Akt pathways (33–35).

In this paper, we report that CD27 activates the Pim kinase pathway. In primed CD8+ T cells, CD27 enhanced Bcl-xL expression but also upregulated expression of the Pim1 gene. Reconstitution of CD27-deficient CD8+ T cells with the Bclxd gene alone or in combination with the McI1 gene did not correct their defective response in vivo, whereas reconstitution with the Pim1 gene partially rescued it. In a clonally expanding CD8+ T cells, CD27 upregulated Pim-1 protein expression and promoted survival in an mTOR- and IL-2–independent fashion. Our data suggest that CD27 sustains survival of primed CD8+ T cells in part via the Pim-1 kinase pathway.

Materials and Methods

Mice

Wild-type (WT), Cd27−/− (36), OT-I (37), Cd27−/−;OT-I, BclxL transgenic (tg) (38), BclxLtg;Cd27−/−, F5 (39), and Cd27−/−;F5 mice on a C57BL/6 background were used for experiments at 7 to 14 wk of age. Mice were of the CD45.2 allosite, unless otherwise indicated. Mice were bred and handled in accordance with national guidelines, and experiments were approved by the Experimental Animal Committee of The Netherlands Cancer Institute (Amsterdam, The Netherlands).

T cell purification

For in vitro cultures, OT-I T cells were enriched from spleen and lymph nodes as described (40), or for the experiment depicted in Fig. 5 by using the Mouse T Lymphocyte Enrichment Set-DM (BD IMag, BD Biosciences, Franklin Lakes, NJ) according to the manufacturer’s protocol. Purity of the resulting T cell populations was checked by flow cytometry using anti-TCR Vβ5.1/5.2 and anti-CD8 mAbs and was always >99%. For microarray purposes, T cells were purified with CD8 MACS beads according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the resulting T cell populations was always >98%.

T cell stimulation in vitro

For in vitro priming of OT-I T cells with artificial APCs (aAPCs), engineered mouse fibroblast lines were used that expresses a minigene encoding the OVA257-264 epitope, which is presented in the context of H-2Kb. These aAPCs were available in two versions, MEC.B7.SigOVA and MEC.SigOVA, that did or did not express CD80 (41). They were retrovirally transduced to stably express mouse CD70 and GFP from a bicistronic plasmid and flow cytometrically sorted to isolate lines with highly comparable levels of H-2Kb expression. These adenovirus aAPCs were seeded at 10^2 cells per well in 24-well plates and cultured overnight. The next day, wells were washed twice with medium to remove any nonadherent cells or cell debris. OT-I or Cd27−/− OT-I T cells (5 × 10^4) were added to the monolayer of aAPCs in 2 ml medium, and plates were centrifuged at 900 × g for 1 min. The nonadherent T cells were removed after 2, 4, 8, or 14 h for microarray or real-time PCR purposes or were gently transferred after 20 h to empty new wells and incubated for an additional 52 h before analysis by Western blot. For in vitro priming of OT-I T cells with anti-CD3 mAbs, cells were cultured in plates that had been precoated with anti-CD3e mAb 145-2C11. For proliferation assays, OT-I T cells were labeled with CFSE and seeded at 1 × 10^6 cells/well in 96-well plates that had been coated with 0.5 μg/ml anti-CD3 eAb. After 72 or 96 h of culture, (live) cell numbers were determined using a CASY cell counter (Scharfe System, Reutlingen, Germany). Propidium iodide (PI) was added, and cell division and death were monitored by flow cytometry. For Pim-1 protein detection, OT-I T cells were seeded at 5 × 10^5 cells/well in 24-well plates that had been coated with 0.2 μg/ml anti-CD3 mAb. As a positive control for Pim-1 activation, cells were stimulated with 10 ng/ml recombinant murine IL-7 (PeproTech, Rocky Hill, NJ). After 6 h or 14 h of stimulation, cells were harvested and processed for Western blot analysis. Where indicated, cells were preincubated with 50 nM rapamycin (Sigma-Aldrich, St. Louis, MO) or vehicle control for 15 min, and rapamycin stayed present during the assay. Cells were stimulated with soluble recombinant FcCD70 (1 μg/ml), which is a fusion protein of the Fe portion of human IgG1 and the extracellular domain of mouse CD70. This fusion protein was generated and validated as described (42). IL-2R signaling was blocked with anti-CD25 mAb PC61 (10 μg/ml).

Western blotting

In vitro-activated OT-I or Cd27−/− OT-I T cells were harvested at the indicated time points and lysed with 1% Nonidet P-40 in 10 mM triethanolamine-HCl pH 7.8, 150 mM NaCl, 5 mM EDTA, and protease inhibitors. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay. Equal amounts of total protein per sample were separated on NuPAGE Novex 8–14% Bis-Tris Gels (Invitrogen), and proteins were transferred to Protran nitrocellulose membranes (Schleicher and Schuell BioScience, Dassel, Germany). The membranes were blocked with 5% powdered nonfat milk (Nutricia, Zoetermeer, The Netherlands) in TBS with 0.05% Tween 20 (TBS-T). Next, membranes were incubated with Abs to Bcl-xL (7B2.5; AbCam, Cambridge, U.K.), Bcl-2 (3F11; BD), McI-1 (rabbit polyclonal; Biolegend, San Diego, CA), BR-1 (rabbit polyclonal; AbCam), survivin (60.11; AbCam), Bim (rabbit polyclonal; Sigma-Aldrich), Bad (clone 48; BD), actin (C4; Millipore, Billerica, MA), or Pim1 (12H8; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T with 1% BSA and 0.1% Tween 20. After three washes with TBS-T, the membranes were probed with peroxidase-conjugated secondary Ab for 2 h in TBS-T with 1% milk at 4˚C. After washing, secondary Ab was detected using ECL (GE Healthcare, Diegem, Belgium).

Retroviral transduction

Retrovirus was obtained by co-transfection of pMX-BclxL-IRES-GFP, pMX-BclxL-IRES-YFP, pMX-BclxL-IRES-YFP, pMX-Mcl-IRES-GFP, pMX-Pim1-IRES-YFP, or pMX-BclxL-IRES-YFP together with pCl-ECO into Phoenix-ECO packaging cells using FuGENE 6 (Roche, Basel, Switzerland). Supernatants containing retrovirus were collected 48 h after transfection. F5 or Cd27−/−, F5 spleenocytes were activated in vitro using 2 μg/ml Con A (Genaplex, Breda, The Netherlands) and 1 ng/ml recombinant IL-7 (PeproTech) for 48 h at 37˚C. For subsequent transduction, they were resuspended in retrovirus-containing medium at 2 × 10^5 cells in 0.5 ml per well in non-tissue culture-
treated 24-well plates (BD Biosciences) coated with 0.5 ml 50 µg/ml RetinoNectin (Takara Bio, Otsu, Japan). Plates were spun for 90 min at 450 x g, and cells were cultured for 20 h prior to adoptive transfer.

Adoptive transfer
For the experiment depicted in Fig. 2, T cells were purified from the spleens of donor mice as described (40), labeled with CFSE, resuspended in HBSS, and injected intravenously at 2.5 × 10^6 cells in 200 µl per recipient mouse. Mice were infected with influenza virus 2 d later. For the experiments depicted in Figs. 3 and 5, splenocytes that had been transduced with retrovirus were incubated with allopurinol-conjugated anti-CD8 mAb for 30 min on ice, in medium with serum. Cells were sorted by flow cytometry for GFP and/or YFP and CD8-allophycocyanin, in medium with serum. The resulting purified transduced CD8+ T cells were suspended in HBSS and injected intravenously at 5 × 10^6 cells in 100 µl per recipient mouse. Mice were infected with influenza virus immediately after T cell transfer.

Virus infection
Influenza virus strain A/NT/60/68 was grown, purified, and tested for hemagglutinin activity and infective titers in the Department of Virology, Erasmus University Rotterdam. Mice were anesthetized and infected intranasally with 30 µl HBSS containing 25 hemagglutinin units of virus to induce primary responses. At the indicated time points, mice were sacrificed, and lungs, spleens, and mediastinal draining lymph nodes (DLNs) of the lungs were harvested. These organs were forced through a nylon mesh to acquire single-cell suspensions. Erythrocytes were lysed on ice for 1 min in 0.14 M NH4Cl, 0.017 M Tris-HCl, pH 7.2. The resulting cell populations were used for flow cytometry.

Flow cytometry
Cells were incubated with specific Abs conjugated to FITC, PE, or allophycocyanin. Allopurinol-conjugated-tetramers of MHC class I H-2D^d H chain, β2-microglobulin, and the influenza nucleoprotein (NP)366-374 peptide (2D11) were prepared as described (36) and used in combination with anti-CD8 mAbs. Cells were analyzed using a FACS-Calibur (BD Biosciences) or CyAn (Beckman Coulter, Brea, CA) flow cytometer in combination with FCS Express (De Novo Software, Los Angeles, CA) or FlowJo (TreeStar, Ashland, OR) analysis software. PI-stained dead cells were excluded from analysis. Monoclonal Abs used for immunofluorescence were anti-CD86 (53-6.7), anti-CD4 (L3T4), anti-CD27 (7BD11, RR3-15). All these Abs were available hybridomas.

Gene expression profiling
Amplified RNA was generated from purified WT and Cd27^−/− OT-I T cells as described (40). Microarrays spotted with the Operon v3 oligonucleotide library were obtained from the central microarray facility of The Netherlands Cancer Institute (http://microarrays.nki.nl). AMO200K oligonucleotide sets were prepared as described (36) and used in combination with anti-CD8 mAbs. Cells were analyzed using a FACS-Calibur (BD Biosciences) or CyAn (Beckman Coulter, Brea, CA) flow cytometer in combination with FCS Express (De Novo Software, Los Angeles, CA) or FlowJo (TreeStar, Ashland, OR) analysis software. PI-stained dead cells were excluded from analysis. Monoclonal Abs used for immunofluorescence were anti-CD86 (53-6.7), anti-CD4 (L3T4), anti-CD70 (FR70), anti-CD80 (16-10A1), anti-CD27 (LG.3A10), anti-CD45.1 (A20), anti-Vβ5.1/5.2 (MR9-4), and anti-Vβ11 (RR3-15). All these Abs were obtained from BD Biosciences or prepared as purified Ig from available hybridomas.

Expression of Bcl-xL, Pim1, and Hprt mRNA was measured in the samples sorted for microarray analysis by real-time PCR (LightCycler 480 Real-Time PCR system; Roche). Fast SYBR Green Master Mix (Applied Bio-systems) was used together with 10 ng cDNA template and 1 µM oligonucleotide primer. The primers used were as follows: Bcl-xL forward, 5′-GGCCCTCCACGTGGAGCCTGGTG-3′, Bcl-xL reverse, 5′-AACCCCTGGTGCTGTCCC-3′; Pim1 forward, 5′-ACGACCTGACGCGCACCAACAG-3′, Pim1 reverse, 5′-GAACCCGCCCTGACCCCAACA-3′; Hprt forward, 5′-CTGGTAAAGACACTCTTGC-3′, and Hprt reverse, 5′-TGAAGTAC-TCATTATAGTCAAGGGCA-3′. Levels of mRNA for the household gene Hprt were used for standardization.

Results

CD27 signaling promotes Bcl-xL expression
To address the mechanism by which CD27 costimulation promotes survival of activated CD8^+ T cells, we first examined whether CD27 regulates expression of Bcl-2 family proteins. For this purpose, an in vitro CD8^+ T cell stimulation assay was used that reliably revealed the prosurvival effect of CD27 (42). In this system, fibroblasts engineered to present OVA257-264 peptide in the context of H-2K^b were used as aAPCs. These aAPCs additionally expressed the CD28 ligand CD80, either alone or together with CD70. WT or Cd27^−/− OT-I TCR tg CD8^+ T cells that recognize the OVA–H-2K^b complex (37) were used as responder cells. In this in vitro model, CD27 engagement resulted in a significant increase in OT-I CD8^+ T cell survival at 72 and 96 h after stimulation (42).

We used comparative settings with the CD27–CD70 axis in place or not by comparing the responses of WT and Cd27^−/− OT-I T cells that had been stimulated with CD70^+ aAPCs or the responses of WT OT-I T cells that had been stimulated with CD70^0 or CD70^− aAPCs. We examined the impact of CD27 signaling on expression of the inhibitory Bcl-2 family members Bcl-xL, Bcl-2, Mcl-1, and Bfl-1, as well as the BH3-only proteins Bim and Bad. In addition, we examined expression of the survivin protein that had been implicated in prosurvival signaling by OX40 (43). Western blot analysis of OT-I T cells isolated at 72 h of culture revealed that Bcl-xL expression was greatly increased in OT-I T cells that had received a CD27 stimulus, whereas expression of Mcl-1, Bim, Bad, or survivin was not affected (Fig. 1A). Expression of Bcl-2 and Bfl-1 was somewhat increased by CD27 signaling in this experiment, but in repeated experiments only Bcl-xL expression was reproducibly and significantly increased in both comparative settings (data not shown). Quantitative real-time PCR indicated that levels of Bcl-xL mRNA consistently increased in response to CD27 signaling at early time points after stimulation (Fig. 1B). These data indicate that in primed CD8^+ T cells, Bcl-xL is upregulated at the mRNA and protein level as a result of CD27 costimulation.

Constitutive Bcl-xL expression does not rescue the survival defect of Cd27^−/− responder T cells in vivo
To examine whether Bcl-xL upregulation was responsible for the prosurvival effect of CD27 costimulation in vivo, we made use of tg mice with constitutive Bcl-xL expression in T cells (38). The Bcl-xL transgene was crossed into the Cd27^−/− line, and the resulting offspring of the indicated genotypes was analyzed. Bcl-xL overexpression in tg naive CD8^+ T cells was validated by Western blotting (Fig. 2A). Purified T cells from WT, Cd27^+/+, BclxLtg, and BclxLtg/Cd27^−/− mice, which were all of CD45.2 alleotype, were labeled with the fluorescent dye CFSE and transferred into WT CD45.1 recipients. Next, these recipients were intranasally infected with influenza virus. CFSE labeling of transferred T cells allowed us to track T cells that had divided and therefore could be defined as responders. The gating strategy for flow cytometric analysis of a spleen sample isolated at day 8 postinfection is shown in Fig. 2B. Total numbers of responding CD8^+CD45.1^+CFSE^- T cells were determined in the lung DLN, spleen, and lung at days 5 and 8 postinfection. In the absence of CD27, accumulation of effector CD8^+ T cells was significantly impaired compared with
that in WT, as demonstrated previously (6, 9, 36). Although tg Bcl-xL expression significantly improved accumulation of WT CD8+ T cells in the spleen and lung, it did not improve the defective accumulation of Cd27−/− CD8+ T cells in DLN, spleen, or lung (Fig. 2C). Similar results were obtained for CD4+ responder T cells (data not shown). These data indicate that constitutive Bcl-xL overexpression is not sufficient to rescue the survival defect of virus-responsive Cd27−/− T cells.

**Combined Bcl-xL and Mcl-1 expression does not rescue the survival defect of Cd27−/− responder CD8+ T cells in vivo**

We considered that Bcl-xL might not be able to counteract the proapoptotic activity of Noxa, a BH3-only protein that has been implicated in the T cell apoptosis following glucocorticoid (15). Therefore, we also examined the potential of Mcl-1 to rescue the survival defect of Cd27−/− responder CD8+ T cells because this antiapoptotic Bcl-2 family member can bind Noxa, whereas Bcl-xL cannot (19). In these experiments, we made use of F5 TCR tg CD8+ T cells that are specific for the immunodominant influenza virus epitope NP366–374 in the context of H-2Db (39) and have been used previously to examine the response of WT versus Cd27−/− CD8+ T cells (6, 42). WT or Cd27−/− TCR tg F5 T cells were retrovirally transduced to express Bcl-xL alone or in combination with Mcl-1, and empty vector-transduced cells served as a reference. Bcl-xL and Mcl-1 overexpression after T cell transduction was validated by Western blotting (Fig. 3A). The different donor T cell populations could be discriminated because the retroviral vectors encoded a bicistronic message enabling coexpression of either GFP or YFP. The transduced and flow cytometrically sorted T cell populations were mixed in a 1:1:1 ratio and injected into the same Cd27−/− recipient mice. The recipient mice were infected with influenza virus. At the indicated days postinfection, cells from lung DLNs, spleens, and lungs were isolated, enumerated, and analyzed by flow cytometry. Representative dot plots of spleen samples show gating on donor T effector cells (CD45.1+) that had diluted CFSE and therefore qualified as antiviral responder cells. C. Absolute numbers of CD8+ responder T cells derived from donor mice were calculated from the percentage of CD8+/CD45.1+/CFSE− cells and the total numbers of cells per organ. Bars represent mean values from three mice per time point (plus SEM). The data shown is representative of three independent experiments. *p < 0.05 (t test).
T cells. WT or \( Cd27^{-/-} \) responder OT-I T cells were stimulated with aAPCs that expressed CD70 and/or CD80 in all relevant combinations with BclxL-ires-GFP only, both BclxL-ires-YFP and Mcl1-ires-GFP, or empty vector (ev)-ires-YFP and mixed in a 1:1:1 ratio. WT or \( Cd27^{-/-} \) F5 T cells were injected into different \( Cd27^{-/-} \) recipient mice, which were subsequently infected with influenza virus and analyzed 8 d later. A, Bcl-xL and Mcl-1 protein expression as shown by Western blotting in transduced \( Cd27^{-/-} \) F5 T cells before adoptive transfer. B, The flow cytometric gating strategy used to enumerate the different transduced F5 T cell populations, as shown for a representative sample from the lung. C, The absolute numbers of transduced F5 T cells in the DLN, spleen, and lung at day 8 postinfection, as calculated from the total number of cells per organ and the percentage of GFP\(^\pm\), YFP\(^\pm\), or GFP\(^+\)YFP\(^+\) CD8\(^+\) T cells. D, The fold increase of WT or \( Cd27^{-/-} \) F5 T cells transduced with BclxL or BclxL plus Mcl1 over WT or \( Cd27^{-/-} \) F5 T cells transduced with the ev control at day 8 postinfection. Bars represent mean values of four mice per time point (plus SEM). The data shown is representative of two independent experiments. * \( p < 0.05 \); ** \( p < 0.01 \) (t test).

\( Cd27 \) upregulates Pim-1 protein expression in primed CD8\(^+\) T cells in a manner independent of mTOR and IL-2R

In murine T cells, Pim-1 occurs in 34- and 44-kDa isoforms that arise from alternative protein translation from the same mRNA template (32). The Pim kinases are regulated by de novo transcription and protein turnover. It was previously found that in murine T cells, Pim-1 protein expression is induced within 30 min after TCR/CD28-mediated T cell activation and persists for at least 12 h (34). To examine whether CD27 signaling upregulated Pim-1 protein expression, we stimulated purified OT-I T cells with a suboptimal amount of coated anti-CD3 mAb, a condition that adequately reveals the prosurvival effect of CD27 (see next paragraph). CD27 was stimulated with an agonistic, recombinant
Data are representative of two independent experiments. 

was added as indicated. Western blotting was performed as described in of mTOR inhibitor rapamycin. Blocking Ab to the IL-2R α-chain (PC61) was added as indicated. Western blotting was performed as described in A. Data are representative of two independent experiments.

soluble form of CD70 (FcCD70) (42). As a positive control for Pim-1 upregulation, we used IL-7 (34). A baseline expression of the 34- and 44-kDa Pim-1 isoforms was observed upon culture of the purified OT-I T cells in medium (Fig. 5A). Under the conditions employed, anti-CD3 mAb alone did not enhance Pim-1 expression, but additional stimulation of CD27 by FcCD70 did. An increase in Pim-1 expression was observed both at the 6-h and 14-h time points after CD27 costimulation.

A contribution of the Pim-1 and Pim-2 kinases to the proliferative response of anti-CD3/CD28-stimulated murine T cells was revealed for the first time under conditions of mTOR inhibition by rapamycin (34). In presence of rapamycin, the activated T cells were fully reliant on the concerted action of Pim-1 and Pim-2 to undergo blastogenesis and to complete successive divisions (34). In presence of rapamycin, CD27 costimulation significantly enhanced the survival of OT-I T cells prior to their first division, as well as throughout successive divisions (Fig. 6).

Under conditions of T cell priming in vitro, cells are generally dependent on IL-2. However in vivo, clonal expansion of activated T cells at the priming site is IL-2-independent, yet supported by CD27 costimulation (42). In presence of blocking Ab to the IL-2R, CD27 significantly incremented live cell yield during blastogenesis and clonal expansion of primed CD8+ T cells. (Fig. 6, Supplemental Fig. 1). This was particularly striking in presence of rapamycin (Fig. 6) and coincided with a pronounced increase in Pim-1 protein expression under these conditions (Fig. 5). We conclude from these findings that CD27 can support the survival of primed CD8+ T cells in a manner independent of the mTOR and IL-2R, most likely via the Pim-1 pathway.

Constitutive Pim-1 expression partially rescues the accumulation defect of Cd27−/− CD8+ T cells in vivo

To examine whether Pim-1 expression was instrumental in the prosurvival effect of CD27 in CD8+ T cells in vivo, we again used the genetic reconstitution approach. WT or Cd27−/− TCR tg F5 T cells were retrovirally transduced with either a Pim1-IRES-YFP vector or with an empty vector-IRES-GFP. The Pim-1 protein expression of the mTOR pathway (34), we tested whether CD27 costimulation was effective under these conditions. OT-I T cells were labeled with CFSE to follow cell division and stimulated with plate-bound anti-CD3 mAb, in presence or absence of FcCD70. At 72 or 96 h after culture, OT-I cells were harvested, and cell division and cell death were determined. Cells were cultured in the presence of rapamycin (Fig. 6) or in its absence (Supplemental Fig. 1). In agreement with Fox et al. (34), we found that TCR-primed CD8+ T cells did enter into cycle and completed several divisions in absence of mTOR signaling (Fig. 6), albeit less efficiently than when mTOR contributed (Supplemental Fig. 1). Importantly, in presence of rapamycin, CD27 costimulation significantly enhanced the survival of OT-I T cells prior to their first division, as well as throughout successive divisions (Fig. 6).

CD27 supports survival of primed CD8+ T cells in a manner independent of mTOR and IL-2R

Because the contribution of the Pim kinase pathway to clonal expansion of primed T cells in vitro was revealed upon inhibition of the mTOR pathway (34), we tested whether CD27 costimulation was effective under these conditions. OT-I T cells were labeled with CFSE to follow cell division and stimulated with plate-bound anti-CD3 mAb, in presence or absence of FcCD70. At 72 or 96 h after culture, OT-I cells were harvested, and cell division and cell death were determined. Cells were cultured in the presence of rapamycin (Fig. 6) or in its absence (Supplemental Fig. 1). In agreement with Fox et al. (34), we found that TCR-primed CD8+ T cells did enter into cycle and completed several divisions in absence of mTOR signaling (Fig. 6), albeit less efficiently than when mTOR contributed (Supplemental Fig. 1). Importantly, in presence of rapamycin, CD27 costimulation significantly enhanced the survival of OT-I T cells prior to their first division, as well as throughout successive divisions (Fig. 6).

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Constitutive Pim-1 expression partially rescues the accumulation defect of Cd27−/− CD8+ T cells in vivo

To examine whether Pim-1 expression was instrumental in the prosurvival effect of CD27 in CD8+ T cells in vivo, we again used the genetic reconstitution approach. WT or Cd27−/− TCR tg F5 T cells were retrovirally transduced with either a Pim1-IRES-YFP vector or with an empty vector-IRES-GFP. The Pim-1 protein expression was instrumental in the prosurvival effect of CD27 in CD8+ T cells in vivo, we again used the genetic reconstitution approach. WT or Cd27−/− TCR tg F5 T cells were retrovirally transduced with either a Pim1-IRES-YFP vector or with an empty vector-IRES-GFP. The Pim-1 protein expression of the mTOR pathway (34), we tested whether CD27 costimulation was effective under these conditions. OT-I T cells were labeled with CFSE to follow cell division and stimulated with plate-bound anti-CD3 mAb, in presence or absence of FcCD70. At 72 or 96 h after culture, OT-I cells were harvested, and cell division and cell death were determined. Cells were cultured in the presence of rapamycin (Fig. 6) or in its absence (Supplemental Fig. 1). In agreement with Fox et al. (34), we found that TCR-primed CD8+ T cells did enter into cycle and completed several divisions in absence of mTOR signaling (Fig. 6), albeit less efficiently than when mTOR contributed (Supplemental Fig. 1). Importantly, in presence of rapamycin, CD27 costimulation significantly enhanced the survival of OT-I T cells prior to their first division, as well as throughout successive divisions (Fig. 6).

Under conditions of T cell priming in vitro, cells are generally dependent on IL-2. However in vivo, clonal expansion of activated T cells at the priming site is IL-2-independent, yet supported by CD27 costimulation (42). In presence of blocking Ab to the IL-2R, CD27 significantly incremented live cell yield during blastogenesis and clonal expansion of primed CD8+ T cells. (Fig. 6, Supplemental Fig. 1). This was particularly striking in presence of rapamycin (Fig. 6) and coincided with a pronounced increase in Pim-1 protein expression under these conditions (Fig. 5). We conclude from these findings that CD27 can support the survival of primed CD8+ T cells in a manner independent of the mTOR and IL-2R, most likely via the Pim-1 pathway.
transcription factor in the lung. Expression of Pim-1 resulted in increased
the absolute number of cells per organ and the percentage of GFP-
DLN, spleen, and lung were quantified. This number is based on
postinfection, the absolute numbers of transduced F5 T cells in the
influenza virus immediately after adoptive cell transfer. At day 8
expression of Bcl-xL, but that expression of Bcl-xL alone or together with Mcl-1 in C27+/−
CD8+ T cells does not correct the survival defect that these cells display during an in vivo immune response. We additionally
demonstrate that Pim1 is a C27 target gene and provide evidence that the Pim kinase pathway acts downstream of C27 to promote
the survival of primed CD8+ T cells in an IL-2–independent manner, most likely by mechanisms that are distinct from in-
hibition of the mitochondrial apoptosis pathway.

The upregulation of Bcl-xL expression after C27 stimulation observed in our study and by others (24) suggested that Bcl-xL is
important for survival signaling by C27. This was all the more
plausible because the closely related costimulatory receptors 4-
1BB and OX40 also upregulate Bcl-xL expression (20–22). We
used Bcl-xL tg T cells as well as T cells that were retrovirally trans-
duced with the Bcl-xL gene to test this possibility. The inclusion of
WT CD8+ T cells in our assays was essential to determine the ca-
pacity of Bcl-xL to correct specifically the deficiency of C27+/−
CD8+ T cells. Constitutive Bcl-xL overexpression improved the survival of both WT and C27+/− responder CD8+ T cells but did
not specifically correct the survival defect of C27+/− CD8+ T cells. Retroviral transduction of OX40-deficient CD4+ T cells
with Bcl-xL reportedly rescued their survival defect in vivo, but
the control of WT T cell transduction was not included in this
study (20). Therefore, it is possible that also in the case of
Ox40−/− CD4+ T cells, Bcl-xL expression alone is not sufficient
to correct for OX40 deficiency. This is supported by the ob-
servation that conditional deletion of the Bcl-xL gene in T cells
did not affect the ability of CD8+ T cells to generate effector
and memory pools in response to Listeria infection (44). The
conclusion of these authors that Bcl-xL is dispensable for these
events is not fully justified, however, because it was not ex-
amined whether compensatory upregulation or downregulation
of other Bcl-2 family members occurred.

Bcl-xL can counteract apoptosis induction via the mitochondrial
pathway by interaction with Bax, Bak, and BH3-only proteins,
which together mediate mitochondrial outer membrane perme-
ability. BH3-only proteins are selective in their interaction with
inhibitory Bcl-2 proteins (13, 19). Although Mcl-1 was not upregulated
in response to CD27 signaling, it was possible that Noxa was
neutralized by inhibitory Bcl-2 family member Mcl-1 (13, 19). Although Mcl-1 was not upregulated
in response to CD27 signaling, it was possible that Noxa was
downregulated, an effect we could not address because the re-
quired reagents to examine Noxa protein expression are lacking.

From gene array experiments, we had no evidence for upregula-
tion or downregulation of mRNA of Bcl-2 family members other
than Bcl-xL. The combined overexpression of both Bcl-xL and
Mcl-1 improved influenza virus-specific CD8+ T cell accumulation
at priming and tissue sites, validating their prosurvival effect.
However, Bcl-xL and Mcl-1 improved the accumulation of WT and C27+/− virus-specific CD8+ T cells to a similar fold over
that of empty vector-transduced T cells that were present in
the same recipients. Therefore, combined constitutive overexpression
of Bcl-xL and Mcl-1 did not compensate for the survival defect of
C27+/− CD8+ T cells. A comparable approach has not yet been
taken to examine the relevance of Bcl-xL and/or Bfl-1 for survival
signaling by 4-1BB and OX40. In repeated experiments, we did not

expression that resulted from T cell transduction was validated by
Western blotting. Both isoforms were strongly expressed (Fig.
7A). These cells were mixed in a 1:1 ratio and injected into
C27+/− recipient mice. The recipient mice were infected with influenza virus immediately after adoptive cell transfer. At day 8
postinfection, the absolute numbers of transduced F5 T cells in the
DLN, spleen, and lung were quantified. This number is based on
the absolute number of cells per organ and the percentage of GFP-
or YFP-positive cells. Fig. 7B shows an example of the gating
strategy used to enumerate the different transduced F5 T cell
populations, as shown for a representative sample from the lung. C
The absolute numbers of transduced F5 T cells in the DLN, spleen, and lung
day at 8 postinfection, as calculated from the total number of cells per
organ and the percentage of GFP−, YFP−, or GFP+/YFP− CD8+ F5 T cells.
D, The fold increase of WT or C27−/− F5 T cells transduced with the
Pim1 gene over WT or C27−/− F5 T cells transduced with the ev
control at day 8 postinfection. Bars represent mean values of six mice per
time point (plus SEM). The data shown is representative of two in-
dependent experiments. *p < 0.05; **p < 0.01 (t test).

FIGURE 7. Retroviral expression of the Pim1 gene partially rescues
the defective accumulation of C27−/− CD8+ T cells. WT or C27+/−
F5 T cells were retrovirally transduced with either Pim1RES-YFP or
empty vector (ev)RES-GFP and mixed in a 1:1 ratio, followed by in-
jection into C27+/− recipient mice. The recipient mice were sub-
sequently infected with influenza virus and analyzed 8 d later. A, Pim-1
protein expression as shown by Western blotting in transfected WT or
C27−/− F5 T cells before adoptive transfer. B, The flow cytomteric
gating strategy used to enumerate the different transduced F5 T cell
populations, as shown for a representative sample from the lung. C
The absolute numbers of transduced F5 T cells in the DLN, spleen, and lung
day at 8 postinfection, as calculated from the total number of cells per
organ and the percentage of GFP−, YFP−, or GFP+/YFP− CD8+ F5 T cells.
D, The fold increase of WT or C27−/− F5 T cells transduced with the
Pim1 gene over WT or C27−/− F5 T cells transduced with the ev
control at day 8 postinfection. Bars represent mean values of six mice per
time point (plus SEM). The data shown is representative of two in-
dependent experiments. *p < 0.05; **p < 0.01 (t test).
find a consistent upregulation of Bcl-2 or Bfl-1 protein (data not shown), but it is possible that these proteins play a part in vivo. However, the binding spectrum of Bcl-2 is similar to that of Bcl-xL, and that of Bfl-1 is similar to that of Mcl-1, and these proteins may compensate for each other upon overexpression (13, 19). Because Bcl-xL or Mcl-1 together are expected to bind to the complete spectrum of BH3-domain–only proteins (13, 19), our data suggested that there is an additional mechanism by which CD27 promotes the survival of primed T cells.

In this context, it was intriguing that we found the *Pim1* gene to be induced very early after CD27 triggering. Given the kinetics of induction, *Pim1* must be a CD27 target gene. Most studies implicate *Pim* gene induction downstream from cytokine receptors as results of Jak–Stat signaling (26). However, *Pim1* gene induction via NF-κB downstream from the TNFR family member CD40 has been reported (46). In addition, BLYS upregulated Pim-1 and Pim-2 in B cells, albeit with late kinetics that do not exclude an indirect effect (47). The *Pim1* gene has originally been defined by insertional mutagenesis as an oncogene that promotes T cell lymphomagenesis (30). Upon mitogenic stimulation, cells prepare for cell division by an increase in cell size (blastogenesis), which requires greatly enhanced macromolecular synthesis. Moreover, to meet the energy demands during rapid cell division, cells become less dependent on oxidative phosphorylation and switch to aerobic glycolysis (29). The Pim kinases play an important role in the functional adaptations that rapid cell division requires. They have multiple activities, including inhibition of Bad-mediated apoptosis, stimulation of glucose uptake and aerobic glycolysis, improvement of the efficiency of translation (26), as well as promotion of gene transcription by histone phosphorylation (48).

The induction of Pim-1 protein expression by CD27 was mTOR independent, in agreement with existing data indicating that Pim kinases provide an mTOR-independent route to support rapidly dividing cells (26). Because Pim-1 transcription in response to CD27 signaling was rapid and transient, whereas Pim-1 protein expression was more sustained, it cannot be excluded that CD27 regulates Pim-1 protein expression also at the posttranscriptional level (e.g., by preventing Pim-1 protein degradation). Additional indirect effects on Pim-1 protein expression can also not be excluded. However, we found that Pim-1 induction by CD27 was independent of the IL-2/IL-2R-2R pathway. CD27 could support the survival and clonal expansion of primed CD8+ T cells independent of the mTOR and IL-2R pathways. This finding ties in with the concept that initial clonal expansion of primed T cells does not rely on IL-2 signaling. Colombetti et al. (49) have shown that TCR/CD28 engagement can drive T cells into cycle and allows them to expand clonally in absence of IL-2 signaling. Moreover, D’Souza and Lefrançois (50) have demonstrated in various models of antigenic stimulation in vivo that IL-2 signaling is dispensable for initiation of CD8+ T cell cycling. In agreement with this, we found in the same influenza virus model as employed in the current study that CD27 supported the survival of virus-specific CD8+ T cells during clonal expansion in the lung DNLs in an IL-2–independent fashion (42). In support, Carr et al. (51) have also documented an IL-2–independent pathway for CD27/CD70-mediated clonal expansion of CD8+ (OT-1) T cells in vitro. Primed CD8+ T cells appeared to require autocrine IL-2/IL-2R signaling for sustenance of late clonal expansion and for their survival as effector cells in nonlymphoid tissue (52). In striking agreement, we found that the *il2* gene is a CD27 target gene in CD8+ T cells, and showed that CD27 stimulated effector CD8+ T cell survival in the lungs of influenza virus–infected mice by inducing autocrine IL-2 signaling (42). The CD27-target genes identified in CD8+ T cells did not include any cell cycle regulators (data not shown), supporting our functional data (6) that CD27 in murine T cells primarily promotes effector T cell accumulation by prosurvival signaling.

The scenario that emerges is one in which cell cycle entry and the completion of successive cell divisions by recently primed T cells at lymphoid sites is IL-2–independent, but, depending on the strength of the TCR signal, relies on CD28 and CD27 costimulation. CD28 amplifies the TCR signal and promotes cell cycle entry by regulating p27kip1 and cyclin expression but also stimulates clonal expansion by antiapoptotic and prometabolic effects that proceed largely via the Akt and mTOR pathways (28, 29, 53, 54). CD27 acts complementarily to CD28 during clonal expansion by providing survival signals that at least in part result from the Pim-1 pathway. Whether the CD28- and CD27-mediated survival signals are fully complementary or partially address the same signaling pathways remains to be established. Future work should address the relative importance of the Pim pathway for the survival of T lymphocytes during the dynamics of the immune response.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplementary figure 1. CD27 promotes the survival of primed CD8+ T cells in presence and absence of IL-2 receptor input.

OT-I T cells were labeled with CFSE and stimulated with a low concentration of coated anti-CD3 mAb, in the presence or absence of soluble recombinant Fc-CD70 as CD27 agonist, either in the absence (control, top panels) or presence of anti-IL-2 receptor mAb (α-IL2R, bottom panels). After 72 or 96 h of culture cells were enumerated, stained with propidium iodide (PI), and subjected to flow cytometry. Results are plotted as the absolute number of live (PI-negative) OT-I T cells that was present in the indicated cell division cycles as defined by CFSE dilution. Data are mean values of three samples + SD and representative of three independent experiments. * P< 0.05, ** P <0.005 (t test).
Supplementary figure 1

[Graph showing data with various conditions and time points.]