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The Pim Kinase Pathway Contributes to Survival Signaling in Primed CD8$^+$ T Cells upon CD27 Costimulation

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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 $\text{Cd27}^{-/-}$ 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 $\text{Cd27}^{-/-}$ 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: 6670–6678.

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 mitochondrial 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 Bad 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 BCL3-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-kB (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).

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 protein assay. Equal amounts of total protein per sample were separated on NuPAGE Novex 4–12% Bis-Tris Gels (Invitrogen), and proteins were subsequently transferred to 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), Mcl-1 (rabbit polyclonal; Biolegend, San Diego, CA), Bfl-1 (rabbit polyclonal; Biolegend, San Diego, CA), Bax (6A6; Abcam), Bad (clone 48; BD), actin (C4; Millipore, Billerica, MA), or Pim-1 (12H8; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T with 0.1% Tween 20 (TBS-T). Western blot analysis showed that indicated Abs were successfully detected in the respective protein samples. Signals were captured by chemiluminescence using an Odyssey system (Luminata Forte; LI-COR).
treated 24-well plates (BD Biosciences) coated with 0.5 ml 50 µg/ml Ret-robNectin (Takara Bio, Otsu, Japan). Plates were spun for 90 min at 450 × 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 × 10⁶ 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 allophycocyanin-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, anti-CD8 mAb for 30 min on ice, in medium with serum. The resulting purified transduced CD8⁺ T cells were suspended in HBSS and injected intravenously at 5 × 10⁶ 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 infectious titers in the Department of Virology, Erasmus University Rotterdam. Mice were anesthetized and infected intranasally with 50 µl HBSS containing 25 hemagglutinin units of virus to induce primary responses. At the indicated time points, mice were sacrificed, and lungs, spleens, and mesenteric 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. Allophycocyanin-labeled tetramers of MHC class I H-2Db H chain, β₂-microglobulin, and the influenza nucleoprotein (NP) 366–374 peptide (mapN26-3) were prepared as described (36) and used in combination with anti-CD8 mAb. 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-CD88 (53–67.7), anti-CD4 (L3T4), anti-CD70 (FR70), anti-CD80 (16-10A1), anti-CD27 (LG3A10), anti-CD45.1 (A20), anti-νβ5.1/5.2 (MR9-4), and anti-β2J1 (RR3-15). All these Abs were obtained from BD Biosciences or prepared as purified Ig from 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 Operator v3 oligonucleotide library were obtained from the central microarray facility of The Netherlands Cancer Institute (http://microarrays.nki.nl). Amplified RNA was labeled using Cy5- and Cy3-ULS (ULS aRNA Labeling Kit; Kreatech, Amsterdam, The Netherlands) and fragmented into stretches of 60–200 nucleotides (nls) 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.

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Quantitative real-time PCR

Expression of Bcl-xL, Pim1, and Hprt mRNA was measured in the samples used for microarray analysis by real-time PCR (LightCycler 480 Real-Time PCR System; Roche). Fast SYBR Green Master Mix (Applied Biosystems) was used together with 10 ng cDNA template and 1 µM oligonucleotide primer. The primers used were as follows: Bcl-xL forward, 5’-GGGCTTCCCC-3’; Pim1 forward, 5’-ACAGCTCTGACCCCAAGC-3’; Pim1 reverse, 5’-GAAGCCACGCTGGCCAACA-3’; Hprt forward, 5’-CTGTTGAAAGGACCTCTGC-3’; and Hprt reverse, 5’TGAAGTAC-TCATTATAGCTAAGGCA-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-2Kᵇ 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-2Kᵇ complex (37) were used as responder cells. In this in vitro model, CD27 engagement results 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⁻ or CD70⁺ aAPCs. We examined the impact of CD27 signaling on expression of the inhibitor 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 protein 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.

Consitutive 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⁻/⁻, Bcl-xLtg, and Bcl-xLtg/Cd27⁻/⁻ mice, which were all of CD45.2 allotype, 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 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\(^+\)-CD8\(^+\) T cells.

**Combined Bcl-xL and Mcl-1 expression does not rescue the survival defect of Cd27\(^+\)-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 glucose deprivation (15). Therefore, we also examined the potential of Mcl-1 to rescue the survival defect of Cd27\(^+\)-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 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\(^+\)/CD8\(^+\) TCR tg F5 T cells were retrovirally transduced to express Bcl-xL alone, or Bcl-xL and Mcl-1 combined, and therefore qualified as antiviral responder cells. A, Bcl-xL 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 ratio and injected into the same recipient mice. The recipient mice were infected with influenza virus immediately after adoptive cell transfer to call the F5 T cells into action, a response that was shown to be highly efficient (6).

This strategy allowed us to examine the effects of constitutive overexpression of Bcl-xL and Mcl-1 in WT or Cd27\(^-\)F5 T cells during an immune response in the same recipient mice. At day 8 postinfection (the peak of the primary response in this model), the absolute numbers of transduced F5 T cells in the DLN, spleen, and lung were determined based on the absolute number of cells per organ and the percentage of GFP- and/or YFP-positive cells. Fig. 3B shows an example of the gating strategy in the lung. Overexpression of Bcl-xL alone, or Bcl-xL and Mcl-1 combined, significantly enhanced the accumulation of both WT and Cd27\(^-\)F5 T cells in all tissues examined (Fig. 3C). However, the accumulation was enhanced to a similar extent for WT and Cd27\(^-\)F5 T cells over their empty vector counterparts was similar in all organs (Fig. 3D). We conclude therefore that, in this well-defined physiological model of influenza virus infection, the combined constitutive overexpression of Bcl-xL and Mcl-1 in Cd27\(^-\)-responder CD8\(^+\) T cells does not suffice to rescue the survival defect that is inherent to their Cd27\(^-\)-genotype.

**Prosurvival protein kinase Pim-1 is a target of CD27 in primed CD8\(^+\) T cells**

To gain further insight into the nature of Cd27 target gene products that contribute to the prosurvival effect of CD27, we performed genome-wide mRNA expression profiling of primed CD8\(^+\) OT-I...
CD27 COSTIMULATION TARGETS THE PIM KINASE PATHWAY

FIGURE 3. Retroviral expression of Bcl-xL alone or combined with Mcl-1 does not rescue the survival defect of CD27−/− responder CD8+ T cells in vivo. WT or CD27−/− virus-specific TCR tg F5 CD8+ T cells were retrovirally transduced with BclxLRES-GFP only, both BclxLRES-YFP and Mcl1RES-GFP, or empty vector (ev)RES-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 fold increase of WT or CD27−/− F5 T cells transduced with BclxL alone or combined with Mcl-1 over WT or CD27−/− F5 T cells transduced with the ev control at day 8 postinfection. Bars represent mean values of four mice per setting of WT versus CD27−/− OT-I cells, the CD27 gene emerged as most strongly and consistently differentially regulated, confirming the validity of the approach (Fig. 4A). This analysis revealed that the Pim1 gene was significantly induced by CD27 signaling at the 2-h time point in all four in vitro conditions (Fig. 4A). To validate this finding, quantitative real-time PCR was performed on the samples of the 2-h time points, which confirmed the results obtained by microarray analysis (Fig. 4B). These data argue that the Pim1 gene is a direct CD27 target and that Pim1 transcription is induced by CD27 signaling at early time points after CD8+ T cell priming.

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 CD3 mAb in presence or absence of Fc-CD70, in the additional presence detect the two Pim-1 isoforms at 34 and 44 kDa. After stripping, the blots were separated by SDS PAGE and subjected to Western blot analysis to upregulation. Cells were lysed at the 6-h or 14-h time point, and lysates they were stimulated with recombinant IL-7 as a positive control for Pim-1 sence of soluble recombinant Fc-CD70 as CD27 agonist. Alternatively, with a low concentration of coated anti-CD3 mAb in the presence or abs- presence of rapamycin (34). Under conditions of mTOR inhibition by rapamycin (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.

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 ex-

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 T 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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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 Cd27−/− recipient mice. The recipient mice were subsequently infected with influenza virus and analyzed at day 8 postinfection. A, 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+, YFP+, or GFP+YFP+ CD8+ F5 T cells. D, The fold increase of WT or Cd27−/− F5 T cells transduced with the Pim1 gene over WT or Cd27−/− F5 T cells transduced with the empty vector control at day 8 postinfection. Bars represent mean values of six mice per time point (plus SEM). The data shown is representative of two independent experiments. *p < 0.05; **p < 0.01 (t test).

FIGURE 7. Retroviral expression of the Pim1 gene partially rescues the defective accumulation of Cd27−/− CD8+ T cells. WT or Cd27−/− F5 T cells were retrovirally transduced with either Pim1-IRES-GFP or empty vector (ev)-IRES-YFP and mixed in a 1:1 ratio, followed by injection into Cd27−/− recipient mice. The recipient mice were subsequently infected with influenza virus and analyzed 8 d later. A, Pim-1 protein expression as shown by Western blotting in transduced WT or 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+, YFP+, or GFP+YFP+ CD8+ F5 T cells. D, The fold increase of WT or Cd27−/− F5 T cells transduced with the Pim1 gene over WT or Cd27−/− 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 independent 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-kB 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 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 Lefrançais (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 DLN s in an IL-2–independent fashion (42). In support, Curr et al. (51) have also documented an IL-2–independent pathway for CD27/CD70-mediated clonal expansion of CD8 (OT-I) 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 p27Kip and cyclin expression but also stimulates clonal expansion by antiapoptotic and prometabolic effects that proceed largely via the Akt and mTOR pathways (28, 29, 49, 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.

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