Molecular mechanisms underlying CD27-CD70 costimulation
Peperzak, L.

Citation for published version (APA):
Peperzak, L. (2010). Molecular mechanisms underlying CD27-CD70 costimulation

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 3

CD27 sustains survival of CTL in virus-infected nonlymphoid tissue in mice by inducing autocrine IL-2 production

Victor Peperzak, Yanling Xiao, Elise A.M. Veraar and Jannie Borst

Journal of Clinical Investigation, 2010
Immunity to infections relies on clonal expansion of CD8+ T cells, their maintenance as effector CTLs, and their selection into a memory population. These processes rely on delivery of survival signals to activated CD8+ T cells. Here, we reveal the mechanism by which costimulatory CD27-CD70 interactions sustain survival of CD8+ effector T cells in infected tissue. By unbiased genome-wide gene expression analysis, we identified the Il2 gene as the most prominent CD27 target gene in murine CD8+ T cells. In vitro, CD27 directed IL-2 expression and promoted clonal expansion of primed CD8+ T cells exclusively by IL-2-dependent survival signaling. In mice intranasally infected with influenza virus, Cd27–/– CD8+ effector T cells displayed reduced IL-2 production, accompanied by impaired accumulation in lymphoid organs and in the lungs, which constitute the tissue effector site. Reconstitution of Cd27–/– CD8+ T cells with the Il2 gene restored their accumulation to wild-type levels in the lungs, but it did not rescue their accumulation in lymphoid organs. Competition experiments showed that the IL-2 produced under the control of CD27 supported effector CD8+ T cell survival in the lungs in an autocrine manner. We conclude that CD27 signaling directs the IL-2 production that is reportedly essential to sustain survival of virus-specific CTLs in nonlymphoid tissue.

Introduction
Control of T cell survival is essential for an adequate immune response (1). Throughout their life span, T cells find themselves in different cellular niches, where they may be resting or proliferating. The proliferative state is particularly stressful, since it may confront the T cell with DNA damage and deprivation of nutrients and/or oxygen, which are circumstances that potentially induce apoptotic cell death (2). In this light, it can be appreciated that various extracellular signals cooperate to regulate T cell survival. To receive these survival signals, T cells are equipped with an array of cell surface receptors that dynamically alters throughout the T cell response. The TCR, cytokine receptors and costimulatory receptors are particularly important in this respect (1–4).

T cell priming takes place in lymph nodes and spleen, which receive the antigen by means of DC delivery. Entry into the G1 phase of the cell cycle requires engagement of the TCR by MHC-peptide complexes (5, 6). This occurs in the context of T cell–DC communication, during which the DC also delivers costimulatory signals. Immature DCs express low levels of CD80 and CD86, which are upregulated upon DC activation (7). CD80 and CD86 are the ligands for CD28, a costimulatory receptor that signals by a similar mechanism as the TCR (5). TCR and CD28 signaling in conjunction set the threshold for T cell entry into S phase and actual cell division (8, 9). Data support the view that CD28 is a signal amplifier required for priming when TCR input is low, but not under conditions of high-dose antigenic stimulation (5, 6). Apart from initiating cell division, CD28 signals counteract apoptosis and regulate cell metabolism (6, 10).

CD28 signaling leads to increased expression of IL-2, a cytokine that is characteristically made by activated T cells (10). IL-2 can drive cell cycling via its multicomponent receptor, which in the high-affinity state consists of a unique α chain (CD25) and β and γ chains that are known to also be part of other IL receptors (3, 11). IL-2 has long been seen as the key mediator of cell cycle progression and T cell clonal expansion. However, TCR and CD28 signaling can also directly drive the cell cycle machinery and allow for IL-2-independent clonal expansion (8, 9, 12). Whereas in vitro systems generally reveal a necessity for IL-2 in clonal expansion of primed CD8+ T cells, it is now clear that the requirements in vivo are distinct (13, 14). In priming lymphoid organs, IL-2 is not required for initial division of CD8+ T cells, but it becomes essential during the late stage of expansion and is particularly important for maintenance of the effector CD8+ T cell pool at tissue sites (12, 15, 16).

Apart from CD28, certain members of the TNF receptor family are important mediators of T cell costimulation, including CD27, 4-1BB, and OX40. TNF receptor family members employ a signaling mechanism that is distinct from the mechanism used by the TCR and CD28. They link to TNF receptor–associated factor (TRAF) family adaptors, which stimulate NF-κB and c-Jun kinase signaling pathways (4). The NF-κB pathway is well known for its antiapoptotic effects, which proceed via transcriptional upregulation of Bcl-2 family members, c-Flip, and inhibitor of apoptosis proteins (17). In addition, these receptors may also activate the antiapoptotic protein kinase B pathway (18). The TNF-related membrane-bound ligands of CD27 and related receptors are expressed on DCs, B cells, and T cells in a transient manner that depends on inflammatory and antigenic signals (4, 19). They therefore typically act in the context of an immune response. Many of the receptors are also induced upon immune activation. CD27, however, is already expressed on naive T cells and plays an important part during T cell priming (20).

Interaction between CD27 and its ligand CD70 is essential for the generation of a CTL effector pool after infection with various viruses, including influenza, vaccinia, and vesicular stomatitis virus (19–22). Upon intranasal infection with influenza virus, CD28 and...
CD27 made an equal contribution to clonal expansion of primed CD8+ T cells in the lung draining lymph nodes (DLNs). Moreover, CD27 was more important than CD28 for the accumulation of CD8+ T cells at the site of infection (20). In this mouse influenza virus infection model, CD27 sustained survival of CD8+ T cells throughout clonal expansion and at the tissue site but did not influence cell cycling (20). The survival signaling induced by CD27 is very potent, since transgenic expression of CD70 on steady-state DCs or B cells induced the (TCR-dependent) conversion of naive CD4+ and CD8+ T cells into effector cells, in the absence of deliberate immunization (23–25). Transgenic expression of CD70 on steady-state DCs could also break CTLA4- and PD1-dependent CD8+ T cell tolerance and convert it into potent antiviral (lymphocytic CMV) and anti-tumor immunity (23). The concept that emerges from this work is that CD27 signals, when superimposed on weak TCR/CD28 signals, keep activated T cells alive and allow them to differentiate into effector cells, whereas they would otherwise be deleted.

To elucidate the mechanism of CD27-mediated prosurvival effects, we employed genome-wide expression profiling of activated CD8+ T cells. We found that Il2 is a key CD27 target gene both in vitro and in vivo and that IL-2 is the mediator of CD27-directed CD8+ T cell survival during clonal expansion in vitro. Using genetic reconstitution of primary T cells and adoptive transfer in an influenza virus infection model, we demonstrated that CD27 mediates the survival of CD8+ effector T cells at the tissue site by stimulating autocrine IL-2 production.

**Results**

CD27-CD70 interactions promote survival of primed CD8+ T cells in vitro.

To define the mechanism of CD27-mediated CD8+ T cell survival, we made use of an in vitro stimulation system. Engineered mouse fibroblasts that present OVA257-264 peptide in the context of H-2Kb (26) were equipped with CD70, CD80, or both and used as artificial APCs (aAPCs) (Figure 1A). As responder cells, H-2Kb/OVA257-264-specific OT-I TCR transgenic T cells were used that were either WT or genetically deficient for CD27.

In this experimental system, OT-I T cells did not expand unless CD28-CD80 interactions were in place (data not shown). In summary, CD27-CD70 interactions significantly increased the survival of primed OT-I T cells, as determined by varying either responder T cells or aAPCs. These results indicated that the experimental sys-
CD27 sustains effector CD8+ T cell survival via IL-2

The previous in vitro data were confirmed and extended in a murine model of acute cardiac allograft rejection. In particular, we found that CD27 was an essential survival factor for CD8+ T cells in vivo. We used a mouse model of cardiac allograft rejection to test the hypothesis that CD27 signaling is required for CD8+ T cell survival in vivo. We found that CD27 signaling was necessary for CD8+ T cell survival in vivo, as shown by the rejection of heart allografts in CD27−/− mice. These results provide evidence that CD27 signaling is a critical survival factor for CD8+ T cells in vivo.

CD27 directs expression of the Il2 gene in primed CD8+ T cells. We performed genome-wide mRNA expression profiling of primed CD8+ OT-I T cells to identify candidate CD27 target genes that might mediate its pro-survival effect. OT-I T cells were stimulated in comparative settings that had only CD27 signaling as a variable. Either WT OT-I T cells were used as responders and CD70 expression on the aAPCs was constant (Figure 2A). The aAPCs were furthermore used in 2 configurations: with and without CD80. In addition, gene expression profiling was performed in a comparative setting of WT and Cd27−/− OT-I T cells that had been primed in vivo (Figure 2B). For this purpose, OT-I mice were immunized intranasally with OVA protein, and 3 or 4 days later, H-2Kb/OVA257-264 tetramer + T cells from mediastinal DLNs and spleen were flow-cytometrically purified. In all cases, the comparative mRNA samples were cohybridized to oligonucleotide arrays that represented 72% of all known mouse genes. CD27-regulated genes were identified on the basis of stringent criteria: they had to be differentially expressed with a P value less than 0.00003 and be found in at least 2 different experimental settings. A heat map was constructed that included in the hierarchy the fold differential expression and the number of experimental protocols in which the gene was found to be differentially expressed. In the comparative setting of WT versus Cd27−/− OT-I cells, the Cd27 (Tnfrsf7) gene emerged as most strongly and consistently differentially regulated, confirming the validity of the approach (Figure 2A). This analysis revealed a set of about 30 potential CD27 target genes (data not shown). This set did not include cell cycle regulators, in agreement with the concept that CD27 — at least in mouse T cells — primarily supports cell survival. The set also did not include defined apoptosis regulatory proteins. Interestingly, the Il2 gene emerged from this analysis as the most prominent CD27 target. It was at the top of the heat map and found in all 5 experimental settings, including the in vivo setting (Figure 2A).

The gene array indicated the differential expression of Il2 mRNA among T cells that had or had not received a CD27 signal but was not an absolute measure. To assess the contribution of CD27 signaling to the total amount of Il2 mRNA produced, we performed quantitative real-time PCR on the mRNA samples of in vitro stimulated OT-I T cells that has also been used for the gene array experiments. The

T cells were used as responders and CD70 expression on the aAPCs was constant (Figure 2A). The aAPCs were furthermore used in 2 configurations: with and without CD80. In this way, we could examine the consequences of CD27 stimulation in the presence or absence of CD28 input and control for possible intrinsic differences between naive WT and Cd27−/− T cells. Gene expression was followed kinetically at 2, 4, 8, and 14 hours after coculture with aAPCs. In addition, gene expression profiling was performed in a comparative setting of WT and Cd27−/− OT-I T cells that had been primed in vivo (Figure 2A). For this purpose, OT-I mice were immunized intranasally with OVA protein, and 3 or 4 days later, H-2Kb/OVA257-264 tetramer + T cells from mediastinal DLNs and spleen were flow-cytometrically purified. In all cases, the comparative mRNA samples were cohybridized to oligonucleotide arrays that represented 72% of all known mouse genes. CD27-regulated genes were identified on the basis of stringent criteria: they had to be differentially expressed with a P value less than 0.00003 and be found in at least 2 different experimental settings. A heat map was constructed that included in the hierarchy the fold differential expression and the number of experimental protocols in which the gene was found to be differentially expressed. In the comparative setting of WT versus Cd27−/− OT-I cells, the Cd27 (Tnfrsf7) gene emerged as most strongly and consistently differentially regulated, confirming the validity of the approach (Figure 2A). This analysis revealed a set of about 30 potential CD27 target genes (data not shown). This set did not include cell cycle regulators, in agreement with the concept that CD27 — at least in mouse T cells — primarily supports cell survival. The set also did not include defined apoptosis regulatory proteins. Interestingly, the Il2 gene emerged from this analysis as the most prominent CD27 target. It was at the top of the heat map and found in all 5 experimental settings, including the in vivo setting (Figure 2A).

The gene array indicated the differential expression of Il2 mRNA among T cells that had or had not received a CD27 signal but was not an absolute measure. To assess the contribution of CD27 signaling to the total amount of Il2 mRNA produced, we performed quantitative real-time PCR on the mRNA samples of in vitro stimulated OT-I T cells that has also been used for the gene array experiments. The
amount of IL-2 mRNA produced was related to the amount of mRNA encoding the household enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) to standardize the mRNA levels. In a setting with aAPCs that did not express CD80, varying CD70 on the aAPCs or varying CD27 on the responder T cells both revealed that CD27 signaling increased IL-2 mRNA production in OT-I T cells, with a gradual increase in IL-2 mRNA levels over time (Figure 2B). In a setting with aAPCs that did express CD80, CD27 signaling also contributed to IL-2 mRNA production (Supplemental Figure 2). However, IL-2 mRNA production by CD27−/− OT-I T cells reached higher levels than when only CD70 was present on the aAPCs, indicating that CD28 signaling also contributed. Under the same conditions and in the same time frame, expression of Ifng or Tnf mRNA was not consistently enhanced by CD27 stimulation, indicating that CD27 specifically directed IL-2 gene transcription (Supplemental Figure 2).

At the protein level, differential production of IL-2 was detected by ELISA in the supernatants of the OT-I T cells that had been stimulated in the in vitro system. WT OT-I T cells produced significantly more IL-2 than CD27−/− OT-I T cells at all time points (Figure 2C). This was not due to higher numbers of WT OT-I T cells, since the differential IL-2 production was also found at early time points prior to cell division (24 and 48 hours). Moreover, it was expressed per 10^6 live cells. These results indicate that CD27+ OT-I T cells were deficient in IL-2 production. WT OT-I cells also expressed higher levels of the IL-2 receptor α chain (CD25) than did CD27−/− OT-I cells at both 72 and 96 hours of culture (Figure 2D). This is in agreement with a documented feed-forward mechanism in which IL-2 upregulates expression of the CD25 gene (11). We conclude that the induction of IL-2 gene expression is a prominent function of CD27 on primed CD8+ T cells.
To analyze the contribution of IL-2/IL-2 receptor signaling to CD27-directed CD8+ T cell survival, we used the in vitro priming system. CFSE-labeled WT and Cd27−/− OT-I T cells were stimulated with aAPCs that expressed CD80 and CD70, and numbers of live OT-I T cells and their division status were read out after 96 hours. As shown above (Figure 1), this system revealed that CD27 signaling promoted the survival of primed OT-I T cells (Figure 4). This survival completely relied on IL-2 receptor signaling, since blocking anti–IL-2 receptor or receptor α-chain mAb PC61 at 10 μg/ml completely abrogated live yield of both WT and Cd27−/− OT-I T cells. There was therefore no pro-survival effect of CD27 revealed in this system that was independent of IL-2 receptor signaling. Lack of CD27 on OT-I T cells did not impair the OT-I T cell response as dramatically as complete IL-2 receptor blocking. This indicates that there was a CD27-independent but IL-2-dependent survival signal in the system, which was most likely due to CD28 signaling. Live Cd27−/− OT-I T cell yield was restored to WT levels upon addition of IL-2, in a dose-dependent manner (Figure 4). The results of this complementation approach support the notion that the survival defect of primed Cd27−/− CD8+ T cells in this in vitro expansion system is due to deficient IL-2 production.

We also examined the mechanism of survival signaling by CD27 using primary DCs as antigen-presenting cells. Conventional DCs were isolated from spleen, loaded with OVA protein, and simultaneously matured with LPS. Subsequently, they were cocultured with CFSE-labeled naive OT-I T cells. This was done in a control setting and in the presence of blocking anti-CD70 or anti–IL-2 receptor antibody. In addition, we included an agonistic soluble recombinant CD70 protein either alone or in combination with anti–IL-2 receptor antibody. At the 72-hour time point, cell division status of live OT-I T cells was read out (Supplemental Figure 3A). This analysis revealed that also in this in vitro stimulation protocol, CD27−CD70 interactions did not affect cell cycle entry or activity, since CD70 blocking or CD27 stimulation with agonistic CD70 did not alter cell division status. However, the same reagents significantly altered live cell yield, with the blocking antibody to CD70 reducing it and the agonistic CD70 protein increasing it (Supplemental Figure 3A). CD70 blocking reduced OT-I T cell survival to a similar extent as IL-2 receptor blocking. Moreover, anti–IL-2 receptor blocking fully abrogated the effect of agonistic CD70. The effects of CD27 stimulation and IL-2 receptor blocking in this model were more modest than when aAPCs were used but were statistically significant. We conclude therefore that also in the context of DCs, survival signaling by CD27 relied on IL-2 receptor signaling.

It was reported previously that — in an in vitro system of OT-I T cell priming in the absence of IL-2—IL-2 receptor interactions — CD27 costimulation prevented downregulation of the IL-7 receptor α-chain (CD127) (28). In our experimental protocol, no impact of CD27 on cell surface expression of the IL-7 receptor or chain was observed (Figure 5A). Addition of recombinant IL-7 enhanced the accumulation of live WT and Cd27−/− OT-I cells, in agreement with a pro-survival effect of this cytokine on primed CD8+ T cells (28). However, in contrast to IL-2, IL-7 did not rescue the survival defect of primed Cd27−/− CD8+ T cells in this in vitro priming system (Figure 5B), underlining the specific role of IL-2 in CD27 costimulation. The collective findings indicate that CD27 mediates survival of primed CD8+ T cells in this in vitro system by stimulating IL-2 production.

IL2 gene reconstitution rescues accumulation of virus-specific Cd27−/− CD8+ T cells at the tissue site. As illustrated in Figure 3A, a key phenotype we have documented previously in Cd27−/− mice is the defective accumulation of primed CD8+ T cells following intranasal influenza virus infection (19–21). Subsequent experiments by various groups have corroborated the importance of CD27/CD70-dependent costimulation in mediating accumulation of CD8+ effector T cells in vivo (22, 29–31). The identification of the IL2 gene as a CD27 target gene and the discovery that IL-2 was fully responsible for the pro-survival effect of CD27 on primed CD8+ T cells in vitro prompted us to test whether it is IL-2 that mediates the CD27-dependent accumulation of effector CD8+ T cells in vivo. For this purpose, we used the F5 mouse strain as donor, since it expresses a transgenic TCR specific for the immunodominant influenza NP366-374 peptide in the context of H-2Db. The IL-2 cDNA was stably expressed in WT or Cd27−/− F5 CD8+ T cells by retroviral transduction, which required in vitro stimulation with ConA and IL-7. The retroviral vector directed bicistronic expression of GFP or yellow fluorescent protein (YFP), which allowed us to flow-cytometrically sort transduced cells prior to adoptive transfer and to track them in vivo. Sorted cells were transferred into Cd27−/− recipient mice, which were subsequently infected with influenza virus. At day 8 after infection, responses were analyzed by flow cytometry and cell counting (Figure 6A).
In one experimental protocol, WT F5 T cells were transduced with an empty vector encoding YFP, and Cd27−/− F5 T cells were transduced with an empty vector encoding GFP. These cells were mixed at a 1:1 ratio and transferred into the same recipient mice. Cd27−/− F5 T cells transduced with a vector encoding IL-2 expressing GFP were transferred into different recipient mice. The gating strategy to identify these populations is shown in Figure 6B. Side-by-side comparison revealed that at day 8 after infection, WT F5 T cells had accumulated to significantly higher numbers in DLN, spleen, and lung of the same recipient mice than Cd27−/− F5 T cells (Figure 6C). This indicated that Cd27−/− F5 T cells had a cell-intrinsic defect that could not be restored by neighboring WT cells. Strikingly, IL-2–reconstituted Cd27−/− F5 T cells accumulated to WT levels in the lung, indicating that IL-2 could correct the accumulation deficit of Cd27−/− F5 T cells in DLN or spleen (Figure 6C). In a second experimental protocol, we compared side-by-side in the same recipients the accumulation of WT F5 cells with empty vector and Cd27−/− F5 T cells that had been reconstituted with the IL2 gene. This analysis corroborated that IL2 gene reconstitution restored the accumulation of Cd27−/− F5 T cells to WT levels (Figure 6D). Next, we performed an essential control, which was to test whether WT F5 T cells profited from IL2 gene transfer to a similar extent as Cd27−/− F5 T cells. Importantly, retroviral IL-2 expression in WT F5 T cells did not alter their accumulation in the lung after influenza virus infection (Figure 6E). This indicated that IL2 gene expression specifically complemented CD27 deficiency. The collective data suggest that the defective accumulation of Cd27−/− effector CD8+ T cells at the tissue site is due to defective IL-2 production.

**Discussion**

In this study, we demonstrate that CD27 instructs primed CD8+ T cells to express IL-2. Provision of IL-2 in the medium corrected the survival defect of Cd27−/− CD8+ T cells in vitro, and genetic reconstitution with the IL2 gene corrected the survival defect of Cd27−/− CD8+ T cells at the tissue site in vivo. These findings indicate that CD27 can promote effector CD8+ T cell survival by directing IL-2 production (Figure 7C). After intranasal infection with influenza virus, expression of CD70, 4-1BB ligand, and OX40 ligand is induced on B cells and DCs in the lung (19). This fits with a role of these receptor-ligand interactions in maintaining effector CD8+ T cells at the tissue site. Indeed, we have documented in this model that the accumulation and maintenance of influenza virus–specific CD8+ T cells in the lung throughout the course of an acute infection relied strongly on CD27 signaling and to a lesser extent on 4-1BB. There was also a contribution by OX40, but this was exerted throughout the contraction or memory phase and impacted only virus-specific memory CD8+ T cells numbers.
CD27 sustains effector CD8+ T cell survival via IL-2

Therefore, among these 3 receptor-ligand systems, the CD27-CD70 system is of the greatest importance for effector CD8+ T cell survival at the tissue site in this physiological mode of virus infection. The mechanism by which the closely related TNF receptor family members CD27, 4-1BB, and OX40 support T cell survival is expected to be similar, given that they employ highly homologous signaling mechanisms (4).

Our data provide a direct connection with the work of D’Souza et al., who studied the relevance of IL-2 production for the primary CD8+ T cell response to viral infection, soluble protein, or tumor antigen in vivo, using T cells from IL-2– or CD25-deficient mice. They showed that regardless of the context of antigen delivery, IL-2 signaling was dispensable for initiation of CD8+ T cell cycling but required for sustained expansion. This requirement was consistently more apparent in nonlymphoid tissue than in secondary lymphoid organs (15). In a follow-up study, they corroborated that autocrine IL-2 production is dispensable for initiation of CD8+ T cell cycling, but required for sustained expansion (16).

Our study reveals that CD27 is a key factor in directing the autocrine IL-2 production that is required for the survival of effector CD8+ T cells in nonlymphoid tissue.

CD27 deficiency compromised survival of virus-specific effector CD8+ T cells in the lung but also impaired their accumulation in DLN and spleen, as documented here and in our previous work (19–21). Reconstitution with the IL2 gene rescued the survival of these cells in the lung, but not in DLN or spleen. Using the reconstitution approach, we could not conclusively test the relevance of IL-2 for survival of CD8+ T cells in the priming lymphoid organs, since the T cells used for adoptive transfer were not naive. They had to be activated in vitro to allow for retroviral transduction. Therefore, their requirements for in vivo survival may be different from those of endogenously primed CD8+ T cells at early stages. Comparing the responses of Cd28–/– and Cd27–/– CD8+ T cells in DLN of influenza virus–infected mice, we have previously found that CD27 contributed to clonal expansion in the DLN to the same extent as CD28. CFSE labeling indicated that CD27 supported survival of primed CD8+ T cells throughout successive divisions after priming (20). Given the available data on the IL-2 independence of initial clonal expansion (13–16), we favor the interpretation that CD27 supports initial clonal expansion in lymphoid tissues via a mechanism other than autocrine IL-2 production. Our in vitro system was fully IL-2 dependent and was therefore not suitable to study such a mechanism. However, Carr et al. have previously documented an IL-2–independent pathway for CD27/CD70-mediated clonal expansion of CD8+ OT-I T cells in vitro that was revealed in presence of IL-7 (28). They found that CD27 stimulated cell division in this system, which we have not observed using adoptive transfer of CFSE-labeled influenza virus–specific CD8+ T cells in vivo (20). There is evidence that it is not IL-7, but possibly rather IL-15 that plays a role in IL-2–independent clonal expansion at early time points after priming (13). It is hoped that in vitro conditions with a cytokine milieu that approximates the situation in lymphoid organs can be used in the future to investigate the alternative mechanism(s) by which CD27 supports the accumulation of antigen-specific T cells in priming organs.

Figure 6
IL-2 rescues survival of virus-specific Cd27+– CD8+ T cells at the tissue site. (A) Strategy: WT or Cd27–/– influenza virus–specific F5 T cells were retrovirally transduced in vitro with a vector encoding IL-2ΔneoGFP or with an empty vector (ev) encoding YFP or GFP only. Transduced F5 T cells were sorted for CD8 and GFP or YFP expression and injected i.v. into Cd27+– mice, which were subsequently infected with influenza virus. At day 8, cells were harvested from DLN, spleen, and lung; enumerated; stained for CD8; and analyzed by flow cytometry. (B and C) Experimental protocol 1: WT F5 T cells with ev-YFP and Cd27–/– F5 T cells with ev-GFP were mixed at a 1:1 ratio and injected into the same recipient mice (B, left). Cd27+– F5 T cells with IL-2ΔneoGFP vector were injected into different recipient mice (B, right). (D) Dot plots show the gating for transduced F5 cells as identified by GFP or YFP expression (in lung). (C) Absolute numbers of YFP+ or GFP+CD8+ of the indicated genotypes based on flow cytometry as outlined in B. (D) Experimental protocol 2: WT F5 T cells with ev-GFP and WT F5 T cells with IL-2ΔneoGFP vector were injected into different recipient mice. Absolute numbers of GFP+ F5 cells are shown. (E) Experimental protocol 3: WT F5 T cells with ev-YFP and Cd27–/– F5 T cells with IL-2ΔneoGFP vector were injected into the same recipient mice. Absolute numbers of GFP+ F5 cells are shown. Data are mean ± SEM of 4 mice per group. *P < 0.05, **P < 0.01 (t test).
Additional work is needed to delineate the intracellular molecular pathways by which CD27 directly or indirectly (via IL-2) can support activated T cell survival. Gene array analysis did not provide obvious clues with regard to regulation of apoptosis regulatory proteins at the transcriptional level at early time points after priming. However, mRNA expression profiling provides only partial insight in the cellular response, since effects at the posttranslational level are not revealed. Ongoing work indicates that CD27 signaling increases the expression of the antiapoptotic protein Bcl-xL, which is in line with findings in human CD4+ T cells (32). From these findings, the authors have proposed a hypothesis that CD27 on CD4+ T cells is essential for the delivery of CD4+ T cell help and directs IL-2 production in CD4+ T cells (43). Moreover, CD27 also directs the expression of MS4A4B, a molecule that we have linked to the “helped” status of memory CD8+ T cells and is instrumental in IL-2 production (43). Other researchers have also found a link between CD27 and IL-2 in memory CD8+ T cell programming. They have demonstrated that CD4+ T cell help is required for maintenance of CD27 expression on memory CD8+ T cells, which facilitated IL-2 expression upon secondary expansion (44).

The collective data indicate that the IL-2 production that is directed by CD27-CD70 interactions plays an important role in promoting CD8+ T cell immunity to viral and tumor challenge. In particular, its role in the maintenance of effector CD8+ T cells in nonlymphoid tissue and its role in the memory response have important implications not only for vaccination strategies in viral infections but also for the treatment of cancer. This is alluded to by the observation that the size of the CD27-CD8+ T cell pool in bulk tumor-infiltrating lymphocytes was positively correlated with tumor regression in melanoma patients (39). This knowledge opens up various avenues for therapeutic intervention.


**Methods**

Mice. WT, Gld−/− (21), OT-I (45), OT-I;Gld−/−, F5 (46), and F5;Gld−/− mice on a C57BL/6 background were used for experiments at 7–12 weeks of age. OT-1 T cells express a transgenic TCR with specificity for OVA257–264 peptide in the context of H-2Dd. They were retransduced to stably express CD70 and GFP from a bicistronic construct and flow cytometrically sorted to isolate lines with highly comparable levels of H-2Dd expression. The OT-1 T cells that had been labeled with CFSE (5 μM), according to the manufacturer’s protocol (Invitrogen), were added at 0.5 × 10^6 per well in culture medium, and plates were centrifuged at 900 g for 1 minute. After 20 hours of coculture, the nonadherent T cells were gently transferred to empty wells and cultured further. At the indicated time points after initial stimulation with the APCs, T cell numbers were determined using a FACScAria (BD) and kept on ice immediately after sorting.

Quantitative real-time PCR. Expression of mRNA for IL-2, IFN-γ, TNF-α, and Hprt were 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 oligonucleotides. The oligonucleotides used to amplify the template DNA were Il2 fwd, 5′-ACGAGCCTTGTGATGGATCACT-3′; Il2 rev, 5′-CCGCAAGGTTCAAGTCTAT-3′; Il2 fwd, 5′-CCCAGACGTC-CAGGGCCAG-3′; Il2 rev, 5′-CCACCACGGAATCGAGGAAG-3′; Tnfα fwd, 5′-CAACGGAGAACGTTCCCGGAC-3′; Tnfα rev, 5′-GAGGGCGCTT-GACCACGAG-3′; Hprt fwd, 5′-CTGTTGAAAGGCTCCTCGC-3′, and Hprt rev, 5′-TGAAGTACTCATTATAGTCAAGGGCA-3′. Levels of mRNA for the household gene Hprt were used for standardization.

ELISA. ELISA was performed in Immuno 96 Well Micro Plates with Maxi-Sorp surface (Nunc). Wells were coated with purified anti-IL-2 mAb JES6-1A12 at 2 μg/ml in PBS as capture antibody. Next, wells were incubated with test culture supernatants. After this, IL-2 was detected with biotinylated JES6-SH4 mAb at 1 μg/ml in PBS with 1% BSA, followed by streptavi- din-conjugated horseradish peroxidase (Sigma-Aldrich) at 1:10,000 in PBS with 1% BSA. The reaction was developed with 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine substrate (Merck) and 0.06% hydrogen peroxide in 0.1 M sodium acetate (pH 5.5) and stopped with 2 M H2SO4. OD450 was read by a Wallac 1420 Victor® multilabel counter (PerkinElmer). A titration curve was created with recombinant murine IL-2 (eBioscience). Incubation steps were performed at room temperature with washing in between.

**Constructs.** Human IL-2 cDNA was obtained from the German Science Center for Genome Research (RGPD) and cloned into the pMXsPAF6GFP vector (48) using BamHI and NotI restriction enzymes. The pCL-Eco vector encoding the eukaryotic retrovirus receptor was obtained from Imgenex Co. CD27 sustains effector CD8+ T cell survival via IL-2

**CD27 sustains effector CD8+ T cell survival via IL-2**

**49**
on ice. Cells were washed and re suspended in IMDM with 2% FCS and sorted by flow cytometry for GFP or YFP and CD8. The resulting purified transduced CD8+ T cells were suspended in HBSS and injected intravenously into each recipient mouse at the indicated cell number in 100 μl. In the experiments in Figure 6, 5 × 10^5 sorted F5 donor cells were injected per experimental group. In the experiment in Figure 7, 5 × 10^5 sorted nontransgenic donor T cells were injected per experimental group.

Antigen challenge in vivo. Mice were infected intranasally with 25 hemagglutinin units of influenza virus strain A/NT/60/68 as described previously (19–21). Alternatively, mice were immunized intranasally with 500 μg OVA protein plus 1 μg cholera toxin (Sigma-Aldrich) in 50 μl HBSS (43).

Expression profiling. RNA extraction, amplification, and hybridization were performed as described previously (43). Microarrays spotted with the Operon v3 oligonucleotide library were obtained from the central microarray facility of The Netherlands Cancer Institute (http://microarrays.knsw.nl). Microarrays were scanned on an Agilent Technologies scanner, and data extraction was done using ImageG 6.0 software (Biodiscovery). Each experiment consisted of 2 microarrays, to allow for dye reversal between the samples, thus reducing systemic errors due to oligonucleotide-specific dye preferences.

The heat map in Figure 2A was created using TIGR (The Institute for Genomic Research) MultiExperiment Viewer software, version 3.1. Genes included in this hierarchy were listed in the analysis when found to be differentially expressed with a P value less than 0.0003 in at least 2 different experimental protocols. Hierarchy in this list was determined by the fold differential expression (M value), ratio of representation (M value), and signal quality. Statistic. Significance in Figures 1–3, 6, and 7 was measured using 2-tailed Student’s t test. Differences were found to be significant when P was less than 0.05.

Acknowledgements

We thank personnel of the experimental animal facility, the flow cytometry facility, and the central microarray facility of The Netherlands Cancer Institute for expert technical assistance; M.J. van Stipdonk for aPCs; P. Schneider for the FcCD70 construct; and J.M. Coquer, T.N.M. Schumacher, and S.H. Naik for critically reading the manuscript. This work was supported by grant 912-04-052 from The Netherlands Organization for Scientific Research (NWO) and grant NKI-2003-2859 from the Dutch Cancer Society.

Received for publication June 15, 2009, and accepted in revised form October 7, 2009.

Address correspondence to: Jannie Borst, Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. Phone: 31-20-5122056; Fax: 31-20-5122057; E-mail: j.borst@nk.nl.


Supplemental figure 1.
Naïve CFSE-labelled WT- or CD27−/− OT-I T cells were co-cultured with aAPC expressing both CD70 and CD80. Cells were stained with TO-PRO-3 and CFSE dilution was determined by flow cytometry at 48 or 72 h. Histograms are representative for n=4.


Supplemental figure 2. CD27 promotes transcription of the il-2 gene in primed CD8\(^+\) T cells.

Quantitative PCR on mRNA samples that were also used for micro-array. Dataset complements that shown in Fig. 2B. WT or CD27\(^{-/-}\) OT-I T cells were stimulated for 2, 4 or 8 h with the indicated aAPC, after which mRNA was extracted. Levels of il-2, ifn\(\gamma\) or tnf\(\alpha\) mRNA were determined by quantitative real time PCR in the indicated samples (same as in A) and related to mRNA levels of the household gene hprt. The numbers represent the fold difference in the levels of mRNA between WT and CD27\(^{-/-}\) OT-I cells. Data are means of two independent reactions. Data in the top left panel are also in Figure 2B (right panel) and are shown here for comparison.
Supplemental figure 3. CD27-mediated CD8$^+$ T cell survival depends on IL-2 receptor signaling.
WT conventional DCs that had been activated with LPS and loaded with OVA were used to stimulate naïve WT OT-I cells. Blocking antibodies (α) to CD70 (FR70) or IL-2 receptor (PC61), or agonistic soluble (s)CD70 protein (48) were added as indicated. After stimulation, cells were enumerated, stained with PI and antibody to CD8 and analysed by flow cytometry. (A) Percentage of live OT-I cells per cell division based on CFSE dilution at 72 h. Data are mean values (+ SEM) for n=3. (B) Absolute number (#) of live OT-I cells at 96 h. T-test indicated significant differences compared to control for the indicated p values.