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Differential Impact of CD27 and 4-1BB Costimulation on Effector and Memory CD8 T Cell Generation following Peptide Immunization

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The factors that determine differentiation of naive CD8 T cells into memory cells are not well understood. A greater understanding of how memory cells are generated will inform of ways to improve vaccination strategies. In this study, we analyzed the CD8 T cell response elicited by two experimental vaccines comprising a peptide/protein Ag and an agonist that delivers a costimulatory signal via CD27 or 4-1BB. Both agonists increased expansion of Ag-specific CD8 T cells compared with Ag alone. However, their capacity to stimulate differentiation into effector and memory cells differed. CD27 agonists promoted increased expression of perforin and the generation of short-lived memory cells, whereas stimulation with 4-1BB agonists favored generation of stable memory. The memory-promoting effects of 4-1BB were independent of CD4 T cells and were the result of programming within the first 2 d of priming. Consistent with this conclusion, CD27 and 4-1BB–stimulated CD8 T cells expressed disparate amounts of IL-2, IFN-γ, CD25, CD71, and Gp49b as early as 3 d after in vivo activation. In addition, memory CD8 T cells, generated through priming with CD27 agonists, proliferated more extensively than did 4-1BB–generated memory cells, but these cells failed to persist. These data demonstrate a previously unanticipated link between the rates of homeostatic proliferation and memory cell attrition. Our study highlights a role for these receptors in skewing CD8 T cell differentiation into effector and memory cells and provides an approach to optimize vaccines that elicit CD8 T cell responses. The Journal of Immunology, 2014, 193: 244–251.
virulent strain of virus, consistent with the higher expression of 4-1BB on activated T cells from mice bearing a higher viral load (13). Although the lack of CD27 signaling results in suboptimal CD8 T cell responses (12, 14–17), deliberate triggering of CD27 by administration of soluble rCD70 (18) or through transgenic expression of CD70 on DCs (19) prevents tolerance induced by injection of a peptide Ag and allows the generation of a population of effector and memory CD8 T cells. Similarly, 4-1BB triggering was shown to prevent peptide-induced CD8 T cell tolerance (20) and augment effector and memory responses following peptide or DC immunization (21–23).

The effects of CD27 and 4-1BB agonists have not been directly compared. We carried out a side-by-side evaluation of the effects of CD27 and 4-1BB triggering on the CD8 T cell response following peptide and protein immunization. Our data reveal key differences in the effects of CD27 and 4-1BB costimulation on the generation of effector and memory CD8 T cells. These data extend our understanding of the mechanisms that regulate CD8 T cell differentiation and have bearing on the use of CD27 and 4-1BB agonists as adjuvants for promoting CTL-mediated immune responses.

Materials and Methods

**Mice and in vivo experiments**

T cells (10^5 or 10^6) from OT-I TCR-transgenic mice were adoptively transferred into C57BL/6 recipients. Mice were challenged i.p. with OVA (Sigma; 5 mg on day 0) and either anti-CD27 mAb [clone AT124 (24); 200 μg] or anti-4-1BB mAb [clone 4B11 (25); 200 μg] on days 0 and 1. Alternatively, mice were primed with 30 nmol OVA peptide 257–264 (day 0) and either recombinant soluble CD70 (18) (250 μg) or 4-1BBL (25) (250 μg) given i.v. on days 0, 1, and 2. Endogenous OVA-specific CD8 T cell responses were generated in C57BL/6 mice by i.v. administration of OVA (5 mg, day 0) and either anti-CD27 or anti-4-1BB mAbs (200 μg, days 0 and 1). To detect secondary responses, primed mice were injected once with OVA peptide 257–264 (30 nmol) and anti-CD40 mAb (clone 3C5; 500 μg). Secondary responses were measured after adoptive transfer of purified (by cell sorting) memory OT-I cells (10^5) obtained 65 d post-immunization (CEDARLANE), and 1BB were transferred into secondary naive recipients. For these experiments, OT-I T cells were enriched using the Mouse CD8 Recovery Kit (Mylabs). The same protocol was applied to lungs, except they were first cut into small pieces. The spleens of mice 4 d postpriming with OVA and either anti-CD27 or anti-4-1BB mAbs (200 μg, days 0 and 1) were dissected and 30–70% Percoll interface after centrifugation (900 × g). The same protocol was applied to lungs, except they were first cut into small pieces.

**Flow cytometry**

Abs against CD8α (53-6-7), CD62L (Mel-14), CD98 (RL388), CD71 (R7217), killer cell lectin–like receptor G1 (KLRG1; 2F1), CD127 (A7R34), CD122 (TM-b1), and perforin (eBioOMAK-D) were purchased from eBioscience. PE-labeled H-2Kb SIINFEKL tetramer was prepared in-house. Anti-CD25 mAb (7D4) was purchased from BD Pharmingen. Intracellular perforin staining was performed using the Foxp3 Staining Buffer Set (eBioscience). For intracellular cytokine staining, cells were activated with SIINFEKL (1 nM) for 4 h at 37°C in the presence of GolgiPlug (BD Pharmingen) prior to surface staining (tetramer and anti-CD8 mAb). Intracellular cytokine staining was performed using BD Cytofix/Cytoperm buffers. Cells were stained with IFN-γ–allophycocyanin (XMG1.2; BD Pharmingen) and IL-2–PE-Cy7 (JES6-5H4; eBioscience). Samples were run on a BD FACSComp II flow cytometer.

**Detection of in vivo proliferation**

BrdU (Sigma; 1 mg) was given i.p. daily for 3 d prior to analysis. BrdU staining was performed according to the manufacturer’s protocol (BD Pharmingen).

**Quantitative real-time PCR**

Total RNA extracted from in vivo–primed OT-I cells and purified by cell sorting was subjected to reverse transcription using Superscript III Reverse Transcriptase, and the resulting cDNA was analyzed by real-time PCR using TaqMan Gene Expression Assays (both from Life Technologies). Data were normalized to hprt expression. Expression assays used were HPRT (Mm00446968_m1), G6pfd (Lilitha) (Mm01614371_m1), T-bet (XMG1.2; BD Pharmingen) and IL-2–PE-Cy7 (JES6-5H4; eBioscience). Samples were run on a BD FACSComp II flow cytometer.

**Statistics**

Statistical significance was determined using the two-tailed Student t test, except for the data in Fig. 2A and 2B, for which a one-tailed test was used.

**Results**

Both CD27 and 4-1BB agonists augment primary CD8 T cell expansion but 4-1BB is superior for establishment of memory

We compared the effects of CD27 and 4-1BB stimulation in vivo on the expansion of adoptively transferred OVA–specific TCR-transgenic OT-I T cells. Injection of OVA alone resulted in an ineffectual primary T cell response. In contrast, coinjection of agonist anti-CD27 mAb or anti-4-1BB mAb with OVA promoted strong expansion of OT-I T cells, as determined by MHC I tetramer labeling of PBMCs (Fig. 1A, Supplemental Fig. 1A) and splenocytes (Fig. 1B). Although the magnitude of OT-I cell expansion following administration of CD27 or 4-1BB mAb was similar in this experimental setting, contraction of the T cell response was more profound following CD27 stimulation (Fig. 1A, 1B). As such, fewer memory OT-I cells were detected following CD27 stimulation in all of the organs examined, including non-nonlymphoid tissues, such as the lungs, liver, and colon (Fig. 1C, Supplemental Fig. 1B). These effects were independent of the initial number of adoptively transferred OT-I T cells. Thus, more memory cells were generated using agonist 4-1BB mAb even when the number of OT-I T cells was reduced by 10-fold (Supplemental Fig. 1C). Furthermore, evaluation of the endogenous OVA–specific CD8 T cell response revealed increased numbers of Ag-specific memory cells in mice primed with OVA and anti–4-1BB mAb compared with those that received OVA and anti-CD27 mAb (Fig. 1D). Moreover, upon rechallenge, the secondary response was significantly higher in the group of mice that were primed with OVA and agonist 4-1BB mAb (Fig. 1E). Memory T cells can be divided into central and effector memory subsets based on expression of L-selectin (CD62L) and the chemokine receptor CCR7. Because the spleen contains both memory subsets (27), we examined whether stimulation via CD27 or 4-1BB exerts differential effects on the generation of central and effector memory cells in this organ. Fifty-four days following immunization, and independent of the type of agonist used, ~80% of OT-I T cells within the spleen displayed high expression of CD44 and CD62L, whereas the remaining ~20% were CD44high but CD62Llow (Supplemental Fig. 1D). These data demonstrate that, although memory T cell generation after CD27 stimulation is curtailed compared with 4-1BB stimulation, the central/effector memory differentiation program remains unaffected.

Additional experiments were conducted using soluble rCD70 and 4-1BBL as agonists instead of mAbs and the OVA 257–264 peptide instead of whole OVA. The frequency of memory cells...
generated using 4-1BBL was significantly higher than that produced by administration of CD70 (Supplemental Fig. 1F, inset). Furthermore, mice primed with OVA peptide and 4-1BBL produced a stronger secondary response than did those primed with OVA peptide and CD70 (Supplemental Fig. 1E). Thus, the differences that we observed are independent of the type of receptor agonist used, regardless of whether it is a mAb or soluble ligand.

**Heightened memory responses following priming with 4-1BB agonist are independent of CD4 T cell help and are the result of higher numbers of resting memory cells**

In some circumstances, CD4 T cell help promotes CD8 T cell priming, as well as the secondary expansion of CD8 memory T cells (28). The ability of CD4 T cells to instill a competency for secondary CD8 T cell expansion is thought to occur during priming. Thus, depletion of CD4 T cells during priming was shown to compromise CD8 T cell memory responses (29, 30). Therefore, we investigated the effects of early CD4 T cell depletion on the ability of CD27 and 4-1BB agonists to prime an endogenous OVA 257–264–specific CD8 T cell response and generate memory. Robust primary and secondary responses were generated in mice that were primed with OVA and an agonist anti–4-1BB mAb in CD4 T cell–depleted or nondepleted mice (Fig. 2A, 2B). Importantly, the memory response generated by priming with agonist 4-1BB mAb was higher than that produced by agonist CD27 mAb, regardless of whether CD4 T cells were absent or present in the mice (Fig. 2A, 2B).

The ability of CD27-generated memory cells to mount a secondary response suggested that the lower memory response produced by this vaccination protocol compared with that using a 4-1BB agonist is unlikely to be due to a defect in secondary expansion. To formally rule out this possibility, memory OT-I T cells generated 2 mo earlier by priming with either OVA and agonist CD27 mAb or OVA and agonist 4-1BB mAb were purified, and equal numbers were adoptively transferred into C57BL/6 recipients. An additional group of mice received an equivalent number of naive OT-I cells. Following rechallenge with OVA 257–264 and agonist anti–CD40 mAb as an adjuvant, the magnitude of CD8 T cell expansion was considerably higher in recipients of memory OT-I T cells compared with those that received naive OT-I T cells, a finding consistent with the higher proliferative and survival capacity of memory T cells (Fig. 2C). Importantly, the magnitude of expansion of the CD27-generated memory cells was similar to that attained by memory cells generated through 4-1BB stimulation. Thus, an increase in the number of resting memory cells in mice primed with the 4-1BB agonist, rather than an enhanced capacity for secondary expansion, accounts for the superior memory response observed with this agonist.

**4-1BB promotes memory precursor cell generation early during the primary response**

CD27 is constitutively expressed on T cells, whereas 4-1BB is expressed transiently on CD8 T cells following engagement of the TCR by Ag and also following stimulation of memory CD8 T cells by IL-15 (13, 31). To investigate at which time point during an immune response 4-1BB influences memory differentiation, we adoptively transferred OT-I T cells into groups of mice and then immunized them with OVA and either agonist CD27 or 4-1BB mAb. Four days after priming, when OT-I cells began to expand, we isolated CD8 T cells using negative-selection columns that ensured that the isolated cells were devoid of CD27 or 4-1BB mAb. Equal numbers of primed OT-I T cells were transferred into secondary recipient mice, and Ag-specific memory CD8 T cells were enumerated 62 d later. In this setting, priming in the presence
of 4-1BB stimulation was again more effective than stimulation via CD27 in generating memory T cells (Fig. 3A). These data demonstrate that 4-1BB triggering by anti–4-1BB mAb beyond the peak of the primary response is not essential for its CD8 T cell memory–promoting effects. To further investigate the time frame during which 4-1BB exerts its programing effect on the generation of memory CD8 T cells, we first analyzed the kinetics of 4-1BB expression on OT-I T cells. 4-1BB was undetectable on naive cells, peaked 1 d after OVA administration, and declined by day 2 (Supplemental Fig. 2A, 2B). In contrast, CD27 was present on naive OT-I T cells, but its expression increased 1 d after priming (Supplemental Fig. 2C, 2D). Fig. 3B shows that the memory-promoting effects of agonist 4-1BB mAb correlated with the kinetics of 4-1BB expression on OT-I T cells. Thus, delaying administration of 4-1BB mAb by 2 d resulted in an accelerated T cell contraction phase that impacted negatively on the generation of the Ag-specific memory pool (Fig. 3B). These data demonstrate that early 4-1BB signaling is required for optimal generation of memory precursor cells. Next, we examined whether coadministration of anti–4-1BB mAb and anti-CD27 mAb rescues the progressive decline in memory CD8 T cells observed with anti-CD27 mAb alone. These experiments showed that, when the two agonists were combined, the magnitude and kinetics of the OT-I cell response resembled that seen with anti-CD27 mAb alone (Fig. 3C). Thus, the CD27-differentiation pathway dominates over the 4-1BB activation pathway, possibly reflecting the constitutive and inducible expression pattern of CD27 and 4-1BB, respectively. Interestingly, agonist CD27 also mediated its costimulatory effects prior to day 2 (Supplemental Fig. 2E). Together, these data show that CD27 and 4-1BB influence the T cell–differentiation program relatively early during T cell activation.

The impact of CD27 and 4-1BB triggering on effector CD8 T cells

CD27 and 4-1BB engagement induced similar proliferation of OT-I T cells when measured on days 2 and 3 postpriming by CFSE dilution or on day 6 by Ki67 staining (Supplemental Fig. 3, data not shown). We then examined whether effector cell generation was differentially regulated by CD27 and 4-1BB signaling. We isolated splenocytes from mice following adoptive transfer of OT-I

FIGURE 2. Secondary expansion of memory CD8 T cells. Kinetics of endogenous OVA-specific CD8 T cell expansion and decline in peripheral blood in intact (A) or CD4 T cell–depleted (B) mice. Mice were primed as described in Fig. 1A and rechallenged on day 55 with OVA peptide and anti-CD40 mAb. (C) Expansion of memory OT-I T cells in peripheral blood following secondary transfer. Equal numbers of resting memory cells generated by OT-I cell transfer and priming with either OVA+anti-CD27 mAb or OVA+anti–4-1BB mAb were transferred into naive recipient mice, followed by injection of OVA peptide and anti-CD40 mAb. An equivalent number of naive OT-I T cells was transferred into an additional group of mice that also was challenged with OVA peptide and anti-CD40 mAb on day 0. Each data point represents the mean ± SE (data are representative of at least two independent experiments with three mice/group). *p ≤ 0.05.

FIGURE 3. The differential effects of CD27 and 4-1BB on memory generation are programmed during priming. (A) Four days following adoptive transfer of naive OT-I T cells and priming with OVA+anti-CD27 mAb or OVA+anti–4-1BB mAb, effector OVA-specific CD8 T cells were isolated from mice, and equal numbers were transferred into secondary recipients. The frequency of OVA-specific CD8 T cells was determined in peripheral blood on day 62. (B) Delaying 4-1BB triggering until day 2 negatively impacts on the generation of memory cells. Following adoptive transfer of OT-I cells and priming with OVA (day 0) or OVA+anti–4-1BB mAb (200 μg) on day 0 or 2, the frequency of OVA-specific CD8 T cells was monitored in blood. (C) The addition of 4-1BB agonist does not rescue the decline in memory OVA-specific CD8 T cells generated through priming with OVA+anti-CD27 mAb. Following adoptive transfer of OT-I cells, the frequency of OVA-specific CD8 T cells was monitored in the blood of mice primed with OVA+anti-CD27 mAb with control rat IgG or anti–4-1BB mAb (200 μg; day 0). Each data point represents the mean ± SE (data are representative of at least two independent experiments with three mice/group). *p ≤ 0.05, ***p ≤ 0.005.
cells and examined perforin expression by intracellular staining and flow cytometry. The frequency of perforin-expressing cells within the OVA-specific CD8 T cell population was higher in mice primed with OVA and anti-CD27 mAb compared with those that received OVA and anti–4-1BB mAb (Fig. 4A, Supplemental Fig. 4A). These findings were verified by additional experiments involving lower numbers of adoptively transferred OT-I T cells (Supplemental Fig. 4B). IL-2 is known to promote CTL effector function (8). Therefore, we examined whether IL-2 is preferentially produced after CD27 costimulation. We found significantly more IL-2–producing OVA-specific CD8 T cells in mice primed with OVA and anti-CD27 mAb compared with those primed with OVA and anti–4-1BB mAb (Fig. 4B, Supplemental Fig. 4C). Furthermore, the frequency of IFN-γ–producing cells was slightly higher on day 3 in mice given agonist CD27 compared with those receiving agonist 4-1BB (Fig. 4C). We also assessed expression of the transcription factors T-bet, comesdermin, Blimp-1, and Bcl-6, which regulate various facets of the effector and memory CD8 T cell–differentiation programs (2), but none displayed differential expression following CD27 or 4-1BB triggering (Supplemental Fig. 4D).

FIGURE 4. CD27 and 4-1BB triggering generates effectors with distinct functional properties. Mice received OT-I cells, followed by OVA alone, OVA+anti-CD27 mAb, or OVA+anti-4-1BB mAb. Direct ex vivo expression of perforin (A) and intracellular cytokine production of IL-2 (B) or IFN-γ (C) in tetramer+ CD8+ T cells after ex vivo restimulation with OVA peptide. Each bar represents the mean ± SE (data are representative of at least two independent experiments with a minimum of five mice/group). **p ≤ 0.01, ***p ≤ 0.005.

Our data show that, compared with 4-1BB, CD27 engagement substantially enhances IL-2 production by CD8 T cells, thus promoting strong autocrine signaling via the IL-2 receptor and skewing differentiation of CTLs toward terminal effectors. Consistent with this notion, priming with OVA and agonist anti-CD27 resulted in higher and more prolonged expression of CD25 (Fig. 5A; day 3), a known target of IL-2 signaling and a component of the IL-2R complex (32). This finding was verified by additional experiments using lower numbers of OT-I T cells (Supplemental Fig. 5A; day 3), a known target of IL-2 signaling and a component of the IL-2R complex (32). To address the role of IL-2 in CTL differentiation after CD27 triggering, we neutralized IL-2 in vivo during priming with OVA and agonist anti-CD27. Neutralization of IL-2 had no effect on accumulation of OT-I cells at the peak of the response on day 6, demonstrating that CD27-mediated expansion of OT-I cells is independent of IL-2 production (Fig. 5D). In contrast, expression of perforin was markedly reduced after IL-2 neutralization (Fig. 5E). Additionally, IL-2 blockade during priming accentuated the death of T cells during the contraction phase, reducing memory T cell numbers (Fig. 5F).

Overall, our data demonstrate contrasting roles for CD27 and 4-1BB costimulation in CD8 effector T cell differentiation, as well as memory generation.

A higher rate of homeostatic proliferation imparted by CD27 signaling is incompatible with long-term maintenance of memory cells

Following Ag clearance, memory CD8 T cells are maintained largely through the action of IL-7 and IL-15 (2). Furthermore, in some models of infection, memory precursor cells can be identified at the peak of the response by increased expression of IL-7Rα and decreased expression of KLRG1 (2). Therefore, we determined whether CD27 and 4-1BB triggering differentially regulate T cell expression of IL-7Rα, the IL-15Rβ subunit (CD122), and KLRG1. Our data reveal that, at early time points and compared with 4-1BB, the downregulation of IL-7Rα on OT-I cells is delayed following CD27 triggering (Fig. 6A). However, when IL-7Rα expression was re-established on memory cells, no obvious differences were detected between the two groups (Fig. 6B). Interestingly, expression of KLRG1 on OT-I T cells initially was induced more efficiently by 4-1BB triggering (Fig. 6A), although at later time points both CD27- and 4-1BB–stimulated cells expressed similar levels of KLRG1 (Fig. 6B). Thus, increased memory formation by 4-1BB costimulation did not correlate with the presence of cells that expressed high IL-7Rα and decreased levels of KLRG-1. Moreover, no differences were detected in the expression of CD122 between the CD27 and 4-1BB groups (Fig. 6B).

To address possible differences in homeostatic proliferation between CD27- and 4-1BB–generated memory T cells, we examined the proportion of cycling memory OT-I cells by measurement of BrdU incorporation. To our surprise, we found that
the proportion of OT-I cells that incorporated BrdU was consistently higher in the CD27 group (Fig. 6C). These data suggest that the waning of CD27-generated memory CD8 T cells is a consequence of a defect in cell survival.

Discussion
In this study, we demonstrated that CD27 and 4-1BB promote generation of effector and memory cells to different extents. Both receptors increase T cell accumulation during the primary response (Fig. 1A, 1B, 1E, Supplemental Fig. 1E); however, engagement of CD27 favors the generation of effector T cells, exemplified by perforin expression (Fig. 4A, Supplemental Fig. 4A, 4B). In addition, the memory T cell pool generated by CD27 triggering is short-lived and gradually wanes with time, which is in sharp contrast with the situation observed following priming with Ag and 4-1BB agonists (Fig. 1, Supplemental Fig. 1E). Consequently, mice primed with Ag and 4-1BB agonists generated a significantly higher secondary response than those given Ag and CD27 agonists (Figs. 1E, 2A, 2B, Supplemental Fig. 1E). It is noteworthy that the inclusion of a CD27 agonist during immunization enhanced the magnitude of the primary and secondary responses compared with peptide alone (Supplemental Fig. 1E), consistent with our previous observation (18). However, by examining memory responses at later time points and conducting a side-by-side comparison with 4-1BB agonists, we discovered that CD27-generated memory CD8 T cells are relatively short-lived compared with those generated by 4-1BB agonists. Thus, targeting 4-1BB during peptide/protein vaccination should provide a more optimal method for generating long-term protective immunity. Whether CD27/4-1BB triggering exerts similar effects on CD8 T cell differentiation...
when using vaccines that induce a higher degree of inflammation remains to be established. The outcome of disrupting the CD27–CD70 interaction on the generation of memory cells has been variable; some, but not all, studies showed a reduction in the number of memory CD8 T cells (12, 35, 36). It was suggested that CD27 signaling promotes the accumulation of MPECs only under conditions in which IL-12 is strongly induced (36), which may explain the conflicting data regarding the effect of CD27 signaling on the generation of memory cells.

Our data demonstrate that continuous triggering of CD27 and 4-1BB during the memory phase is not required for the differences that we observed in the longevity of memory cells generated by CD27 or 4-1BB agonists (Fig. 3, Supplemental Fig. 2). These data suggest that the memory cell outcome is predetermined during the priming phase. We provide evidence for this notion by demonstrating differences in CD25 expression on CD8 T cells as early as 3 d after priming with CD27 and 4-1BB agonists (Fig. 5A, Supplemental Fig. 4E). Prolonged expression of CD25 on a subset of activated CD8 T cells during acute viral infection was shown to mark a population of cells with enhanced effector function and inability to mature into functional long-lived memory cells (7). Therefore, our data identify CD27 as a key driver of sustained CD25 expression on activated CD8 T cells. In addition to CD25, the transferrin receptor (CD71) and Gp49b (34, 37) were preferentially induced in CD27-stimulated CD8 T cells (Fig. 5B, 5C). These molecules, together with perforin, are known to be upregulated by IL-2 (8, 32–34). Because IL-2 production by CD8 T cells is induced more strongly by CD27 triggering compared with 4-1BB (Fig. 4B), our data suggest a scenario in which differentiation toward cytotoxic effectors is regulated in a CD8 T cell-autonomous manner through CD27 and IL-2 signaling (Fig. 5E). However, not all of the costimulatory effects of CD27 require IL-2; early expansion and accumulation of OT-I T cells at the peak of the primary response occurred independently of IL-2 (Fig. 5D). Whether the skewed differentiation of CD27-stimulated CD8 T cells into short-lived memory cells is mediated by the increased production of IL-2 could not be discerned. IL-2 neutralization did not restore stable memory but instead accentuated the contraction of the T cell response (Fig. 5F), suggesting that IL-2 maintains the survival of short-term memory cells (9). Our data are consistent with the notion that CD27 triggering skews the CD8 T cell–differentiation program toward terminal differentiation. Although in this study we used enforced costimulation by administering agonists, a setting pertinent to vaccination, other investigators showed that the lack of CD27 can compromise IL-2 production by influenza virus–specific CD8 T cells (38). This suggests that the effects of CD27 agonists on IL-2 production reported in this article resemble the effects of the endogenous CD27–CD70 interaction.

IFN-γ is a potential factor that contributes to the decline in memory after priming with CD27 agonists because it is elevated in CD27-stimulated T cells both after acute stimulation (Fig. 4C) and in transgenic mice that constitutively express CD70 (39). Thus, IFN-γ-deficient mice have normal expansion of Ag-specific CD8 T cells postinfection with attenuated Listeria monocytogenes but reduced contraction, culminating in the persistence of elevated numbers of Ag-specific CD8 T cells (5, 40).

Although in certain infection models memory precursor cells can be identified by increased expression of the IL-7Rα subunit and decreased expression of KLRG-1 (2), this was not the case in our vaccination protocol (Fig. 6A, 6B). In contrast, prolonged expression of CD25 was a more informative predictor of cells that differentiate into short-term memory cells, thus corroborating previous findings obtained in the acute lymphocytic choriomeningitis virus infection model (7). Additionally, our data highlight increased expression of CD71 and Gp49b as potentially useful in identifying short-term memory precursor cells. The general applicability of these markers in distinguishing memory precursor subsets during infection requires further attention.

The differential effects elicited by CD27 and 4-1BB agonists during priming on homeostatic memory cell proliferation are intriguing and indicate that the relative lack of memory in CD27-primed mice is due to a higher rate of cell death. CD8 T cell death during the contraction phase is controlled by the Bcl-2–regulated “intrinsic” death pathway, as well as the “extrinsic” death pathway that is dependent on CD95 (41). Using a previously established protocol (42), we administered a neutralizing anti-CD95 ligand mAb to address whether CD95 signaling contributed to T cell death in our model. Administration of anti-CD95 ligand mAb did not affect OT-I T cell death following priming with OVA and agonist anti-CD27 (data not shown), consistent with the lack of CD95 involvement reported in other studies of acute CD8 T cell responses (41). In contrast, transgenic overexpression of Bcl-2 in OT-I T cells [vav-bcl-2 × OT-I mice (43)] markedly enhanced T cell survival at the peak of the response and during the contraction phase, independent of the type of agonist used for priming. Nevertheless, 4-1BB–generated memory T cells still exhibited better long-term survival than did memory cells generated by priming with an agonist anti-CD27 (data not shown). The inability of Bcl-2 to restore CD27–generated memory cell frequencies to levels similar to those produced after priming with the 4-1BB agonist could be due to insufficient sequestration of proapoptotic Bcl-2 family proteins. Alternatively, it may be that expression of the Bcl-2 transgene is sufficient to neutralize proapoptotic Bcl-2 family proteins, but cell death ensues through a nonapoptotic programmed cell death pathway (44). Moreover, it is possible that CD27-generated memory cells, which proliferate more extensively than do memory cells generated with a 4-1BB agonist (Fig. 6C), die because they cannot sustain their bioenergetic needs. Because memory T cells generally rely on oxidative phosphorylation for their bioenergetic requirements compared with effector cells, which primarily use glycolysis (45, 46), a reduced mitochondrial mass and, thus, lower mitochondrial spare respiratory capacity in CD27-generated memory cells, could lead to their rapid demise.

In summary, we showed that an early and narrow window of CD27 or 4-1BB triggering during priming skews the differentiation program of Ag-stimulated CD8 T cells, such that effector function and memory cell numbers are differentially affected. Both receptors recruit TNFR-associated factors through short peptide motifs as a way of activating a largely shared set of signaling pathways (11). However, differences may exist in the magnitude and duration of signaling as a result of subtle changes in the kinetics of binding of TNFR-associated factors to specific receptors that could translate into functional differences. Taken together, our study identifies a novel approach for regulating effector and memory cell generation and, thus, a strategy to optimize vaccines that elicit CD8 T cells.

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Disclosures
The authors have no financial conflicts of interest.
Supplemental Figure 1. (A) Representative flow cytometry plots of H2-Kb OVA tetramer and CD8 staining of blood samples (day 16) following priming as described in Fig.1A. (B) Numbers of OVA-specific T cells isolated from spleen and liver on day 57 following priming as described in Fig.1C. Data points represent the mean ± SE (data are representative of at least 2 independent experiments, n = 4 mice/group). (C) Frequency of OVA-specific T cells in blood during the effector (day 6) and memory (day 35) phases following adoptive transfer of 1x10^4 OT-I T cells and priming with either OVA+anti-CD27 or OVA+anti-4-1BB. Data points represent the mean ± SE (data pooled from 2 independent experiments, n = minimum of 6 mice/group). (D) CD62L expression by Ova-specific CD44hi CD8 memory T cells obtained from the spleen on day 54 following OT-I T cell transfer and subsequent challenge with either Ova+anti-CD27 or Ova+anti-4-1BB. (E) Frequency of OT-I T cells in blood during the primary and secondary response (inset shows the frequency of resting memory OT-I cells - day 62). Following adoptive transfer of 1x10^5 OT-I T cells mice were primed with OVA peptide+control human IgG1, OVA peptide+ soluble CD70, or OVA peptide+soluble 4-1BBL and re-challenged on day 63 with OVA_257-264 peptide and agonist anti-CD40 mAb. Data shown are representative of a minimum of 3 mice per group. *P ≤0.05, **P ≤0.01.
Supplemental Figure 2. Expression of 4-1BB (A-B) and CD27 (C-D) on OVA-specific T cells. Following adoptive transfer of Thy1.1 OT-I CD8 T cells and priming with OVA, splenocytes were labeled with Thy1.1, CD8, CD27, 4-1BB or isotype-matched control mAb. Representative plots from naïve and day 1 OT-I cells are shown in (A) and (C), isotype control staining indicated by the shaded histogram, 4-1BB or CD27 staining are shown as black lines. Data are representative of 3 mice. Corrected MFI (isotype subtracted) kinetics of 4-1BB (B) or CD27 (D) are shown. (E) Delaying CD27 triggering until day 2 negatively impacts on priming. The frequency of OVA-specific CD8 T cells was monitored in blood following the protocol described in Fig.3B. Data points represent the mean ± SE (data are representative of at least 2 independent experiments, n=3 mice). *P ≤0.05, ***P ≤0.005.
Supplemental Figure 3. CD27 and 4-1BB triggering promote similar proliferative responses. Mice received 10^5 CFSE labelled OT-I cells prior to priming with OVA, OVA+anti-CD27 or OVA+anti-4-1BB. Splenocytes were isolated 3 days later and CFSE levels were determined in OVA-specific CD8 T cells. Numbers indicate the % of CFSE<sup>lo</sup> OT-I T cells. Data are representative of a minimum of 3 mice/group.
Supplemental Figure 4. (A) Representative plots of perforin staining of OT-I T cells obtained from unimmunized (naive) mice (with 1x10^5 adoptively transferred OT-I cells) or day 3 primed mice. (B) Frequency of perforin+ OVA-specific T cells isolated on day 4 following adoptive transfer of 1x10^4 OT-I T cells and priming with OVA, OVA+anti-CD27 or OVA+anti-4-1BB. Data points represent the mean ± SE (n = 3 mice/group). (C) Representative plots of intracellular IL-2 staining of OT-I T cells obtained from naive mice (with 1x10^5 adoptively transferred OT-I cells) or day 3 primed mice - shaded histograms represent IL-2 staining of unstimulated cells; black lines represent IL-2 staining of OVA peptide stimulated cells. Data are representative of at least 3 mice/group. (D) Relative expression levels of T-bet, Eomes, Blimp1 and Bcl-6 mRNA in OT-I T cells isolated at the peak of the primary response. Each bar represents the mean ± SD (2 biological replicas with 2 mice per group). *P ≤0.05, **P ≤0.01. (E) Frequency of CD25hi OVA-specific T cells isolated on day 4 following adoptive transfer of 1x10^4 OT-I T cells and priming with OVA, OVA+anti-CD27 or OVA+anti-4-1BB. Data points represent the mean ± SE (n = 3 mice/group). **P ≤0.01.