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Chapter 6

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Submitted
OX40 and 4-1BB, together with CD27, determine the amount of CD8\(^+\) memory T cells formed and imprint into them the capacity for secondary expansion.

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Summary
We have established the relative impact of CD27-, OX40- and 4-1BB receptor/ligand interactions on the CD8\(^+\) T cell response to influenza virus. CD27 and to a lesser extent 4-1BB promoted accumulation of CD8\(^+\) effector T cells during primary infection, while OX40 did not. Nevertheless, all three receptor/ligand systems equally and critically contributed to the formation of memory CD8\(^+\) T cells. Moreover, they collectively determined the magnitude of effector CD8\(^+\) T cell accumulation after secondary infection. Surprisingly, wild-type CD8\(^+\) memory T cells did not require stimulation by OX40- or 4-1BB ligand throughout the secondary response. However, they were impaired in maintenance and capacity for secondary expansion, when they had been generated in OX40- or 4-1BB ligand-deficient mice. Thus, stimulation of CD8\(^+\) T cells during the primary response by OX40- and 4-1BB ligand presented on non-T cells endows them with the capacity for long-term survival and imprints their potential for secondary expansion.

Introduction
An effective immune response relies on the clonal amplification of antigen-specific T cells and their accumulation at the site of infection. For long-term protection, part of the antigen-specific T cell pool must be retained as memory cells, which respond rapidly to renewed challenge. Naïve T cell expansion is initiated by TCR signaling, but this alone is not sufficient. Costimulatory receptors must be engaged, which promote T cell division and survival and may direct development of effector functions. Costimulatory receptors comprise immunoglobulin superfamily members, including CD28 and ICOS, and TNF receptor family members, such as CD27, OX40 and 4-1BB (Watts and DeBenedette, 1999; Croft, 2003). Like the TCR, CD28 and relatives signal via tyrosine kinases, while costimulatory TNF receptor family members signal via TRAF adaptors (Gravestein and Borst, 1998). Both mechanisms activate the NFkB and Jun kinase pathways, posing the
question whether signals provided by the various costimulatory receptors merely add to TCR signaling and to each other in quantitative terms.

Previously, we have determined that CD27 gives a critical survival signal to activated T cells (Hendriks et al., 2003). Upon intranasal infection with influenza virus, CD27 and CD28 equally contributed to expansion of virus-specific T cells and their accumulation at the effector site. CD27 and CD28 appeared complementary in qualitative terms, i.e. CD27 did not promote cell division, as well as in the timing of their pro-survival effects. Like CD28 (Okkenhaug et al., 2001), CD27 probably counters apoptosis by transcriptionally upregulating inhibitory Bcl-2 family members (Van Oosterwijk and Van Lier, pers. comm.).

OX40 and 4-1BB, the closest relatives of CD27 (Croft, 2003), also promote activated T cell survival by stimulating expression of anti-apoptotic Bcl-2 family members, such as Bcl-x<sub>L</sub> and Bfl-1 (Rogers et al., 2001; Lee et al., 2002). While this mechanism of action suggests redundancy between CD27, OX40 and 4-1BB, complementarity may lie in the timing of their involvement in the T cell response. The expression patterns of these receptors give a clue that this might be the case. CD27 is expressed on naïve, effector and memory T cells (Lens et al., 1998; Gravestein et al., 1995), but OX40 and 4-1BB are acquired at the effector stage. They are induced by TCR signals, with CD28 enhancing the kinetics and levels of expression (Al-Shamkani et al., 1996; Gramaglia et al., 1998; Rogers et al., 2001; Pollok et al., 1993). Accordingly, in activated T cells lacking OX40 or 4-1BB signaling, survival is not compromised initially, but it is defective in the later divisions (Rogers et al., 2001; Cooper et al., 2002).

The ligands are TNF-related transmembrane molecules, which emerge transiently, under strict control of antigen receptors and Toll-like receptors (Gravestein and Borst, 1998). CD70, the ligand of CD27, OX40 ligand (OX40L) and 4-1BB ligand (4-1BBL) have all been found on activated T- and B lymphocytes and mature DC (Oshima et al., 1998; Tesselaar et al., 2003a; Al-Shamkani et al., 1996; Stuber et al., 1995; Futugawa et al., 2001), indicating that receptor/ligand interactions come into play during communication between T cells and antigen presenting cells as well as amongst effector T cells. Limited data are available on their expression in vivo. Deliberate constitutive expression of CD70, OX40L or 4-1BBL by transgenesis upsets lymphocyte homeostasis, leading to immunodeficiency or auto-immunity, indicating that the transient availability of these ligands is crucial to prevent pathogenesis (Arens et al., 2001; Tesselaar et al., 2003b; Murata et al., 2002; Zhu et al., 2001).

OX40 and OX40L-deficient mice have reduced primary CD4<sup>+</sup> T cell responses to several viruses and common protein antigens (Kopf et al., 1999; Chen et al., 1999; Murata et al., 2000). Lower frequencies of antigen-specific effector CD4<sup>+</sup> T cells are generated late in the primary response and fewer CD4<sup>+</sup> memory T cells develop (Gramaglia et al., 2000).
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In mice lacking 4-1BBL, fewer antigen-specific CD8+ effector T cells and memory T cells develop (Tan et al., 1999; DeBenedette et al., 1999; Bertram et al., 2002). In CD27−/− mice infected with influenza virus, accumulation of both CD4+ and CD8+ effector T cells is reduced (Hendriks et al., 2000). Phenotypes of receptor- and ligand deficient mice are subtle as compared to the phenotypes of mice in which the receptors are deliberately stimulated. Agonistic anti-OX40- or 4-1BB antibodies rescued T cells from activation-induced cell death and promoted memory formation (Maxwell et al., 2000; Takahashi et al., 1999). In CD70 transgenic mice, effector CD4+ and CD8+ T cells develop in the absence of deliberate antigenic challenge (Arens et al., 2001; Tesselaar et al., 2003b) and in non-immunized OX40L transgenic mice, CD4+ effector T cells accumulate. In addition, in OX40L transgenic mice CD4+ effector T cells showed decreased contraction after antigenic challenge (Murata et al., 2002).

There are many data on the role of costimulatory receptors in primary responses, but much less is known about their importance for memory responses. We have found in the influenza virus model, that accumulation of virus-specific CD8+ effector T cells in the memory response is equally and critically dependent on both CD27 and CD28 (Hendriks et al., 2000; 2003). Intervention with OX40-Ig, as well as testing the performance of OX40−/− T cells in a model of virus-induced lung inflammation indicated that OX40 controls the accumulation of CD4+ effector cells in the memory response (Humphreys et al., 2003; Salek-Ardakani et al., 2003). In mice lacking 4-1BBL, accumulation of CD8+ effector T cells upon re-challenge with virus is reduced (Bertram et al., 2002). These data indicate that CD27 and 4-1BB are important for CD8+ memory responses and OX40 for CD4+ memory responses, but do not elucidate whether this is due to an effect on memory cell formation and/or responsiveness.

We wondered whether CD27, OX40 and 4-1BB make complementary contributions to the same CD8+ T cell response. We therefore infected recombinant mice lacking relevant receptors or ligands with influenza virus and analysed the generation of CD8+ effector T cells, their conversion into memory T cells and memory cell responsiveness. We also monitored expression of receptors and ligands in lymphoid organs and lung throughout infection. Our results highlight the complementarity between these receptor/ligand systems and revealed that OX40 and 4-1BB can endow CD8+ T cells with the capacity for secondary expansion during the primary response.

Results
Primary T cell responses to influenza virus in the absence of CD27, OX40L or 4-1BBL
Intranasal challenge with influenza A virus NT/60/68 characteristically causes a dramatic influx effector T cells into the lung. Wild-type, CD27−/−, OX40−/− or 4-1BBL−/− mice were sacrificed at days 6, 8, 10 or 14 after infection and cell suspensions were extracted from
Figure 1. Primary CD8+ T cell responses to influenza virus in mice deficient for CD27, OX40L, or 4-1BBL.

Wild-type (WT), CD27−/−, OX40L−/−, and 4-1BBL−/− mice were infected intranasally with influenza virus. At indicated days after infection, cells from lungs, DLN and spleens were isolated, counted, stained with anti-CD8 mAb and H2-Db/NP366-374 tetramers and analyzed by flow cytometry. Absolute numbers of cells were calculated from the percentage positive T cells and the total number of cells isolated. (A) Size of total CD8+ T-cell infiltrates in lung. (B) Numbers of H-2Db/NP366-374+ CD8+ T cells in lung, DLN and spleen. Bars represent mean values from 4 mice per time point, error bars show standard error of the mean. Two-tailed Students T-test indicated significant differences compared to wild-type values for p< 0.05 (*) and p<0.01 (**). The experiment is representative of two.

In lungs, mediastinal lung-draining lymph nodes (DLN) and spleen. In wild-type mice, an influx of CD8+ T cells into the lungs had taken place at day 6, peaked at day 8-10 and declined at day 14 (Fig. 1A). Absence of CD27 dramatically reduced the numbers of CD8+ T cells accumulating in the lung. In contrast, deletion of OX40L or 4-1BBL did not affect the size of the CD8+ T cell infiltrate in the lung in a statistically significant manner (Fig. 1A). To follow virus-specific CD8+ T cells, we used MHC H-2Db tetramers loaded with the immunodominant NP366-374 peptide. In wild-type mice, H-2Db/NP366-374+ CD8+ T cells appeared in the lung at day 6 after infection, reached peak numbers at day 8-10 and declined thereafter (Fig. 1B). In CD27−/− mice, the H-2Db/NP366-374-specific T cell response was significantly reduced in DLN and lung, as documented previously (Hendriks et al., 2000, 2003). OX40L deficiency did not affect the accumulation of CD8+ virus-specific T cells in these organs. In absence of 4-1BBL, the CD8+ T cell response in DLN and lung was significantly reduced, but to a lesser extent than in absence of CD27. In the spleen, none of
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The receptor/ligand systems made a significant contribution to the ultimate accumulation of virus-specific CD8+ T cells.

We conclude that upon primary infection with influenza virus, CD27 makes the most important contribution to generation of effector CD8+ T cells in DLN and the accumulation at the site of infection. The interaction of 4-IBB with its ligand also promotes accumulation of CD8+ virus-specific T cells in DLN and lung, but to lesser extent than CD27. OX40 receptor/ligand interaction, however, does not make a detectable contribution to the primary CD8+ T cell response.

Memory T cell responses to influenza virus in the absence of CD27, OX40L or 4-1BBL

To compare the capacity of mice deficient for CD27, OX40L and 4-1BBL to mount a memory CD8+ T cell response, wild-type and recombinant mice were re-infected with the same virus 6 weeks after the first challenge. Since the antibody response to this virus is not affected in the recombinant mice (Xiao et al., 2004), this does not differentially affect viral load. The occurrence of H-2D^b/NP_366-374+ CD8+ T cells was determined 3, 5, 7 and 11 days after secondary infection. Characteristically, H-2D^b/NP_366-374+-specific T cells were detectable in the lungs of wild-type mice earlier than in the primary response (at day 3 versus 6), reached peak levels earlier (days 5 versus 8-10) and were higher in number at the peak of the response (Fig. 2). Strikingly, in this secondary response all three receptor/ligand systems were required for accumulation of H-2D^b/NP_366-374+-specific T cells in the lung. In

Figure 2. Memory CD8+ T cell responses to influenza virus in mice deficient for CD27, OX40L, or 4-1BBL. Six weeks after primary infection, wild-type (WT), CD27-, OX40L-, 4-1BBL-, mice were re-infected. Cells were harvested at the indicated time points and analyzed as outlined for Fig. 1. Numbers of H2-D^b/NP_366-374+ CD8+ T cells in lung, DLN and spleen. Bars represent mean values from 4 mice per time point. Statistical analysis was done as for Fig. 1. The experiments are representative of two.
DLN and spleen, generation of H-2D\textsuperscript{b}/NP\textsubscript{366-374}+memory effector T cells was also reduced, but to a lesser extent than their accumulation in the lung. CD27 deficiency had the most profound impact, while lack of 4-1BB had some effect and lack of OX40L had almost no statistically significant consequences (Fig. 2).

The data demonstrate that memory CD8\textsuperscript{+} T cell response to intranasal influenza virus infection critically depends on the collective, partially non-redundant contributions that result from interaction between CD27, OX40, 4-1BB and their ligands.

**Expression of receptors and ligands on T cells and antigen-presenting cells during anti-viral responses**

To understand where and when throughout the immune response to influenza virus CD27, OX40, 4-1BB and their ligands might interact, we performed an extensive analysis of their expression. At different time points after infection, cells were isolated from lung, DLN and spleen and double stained with antibody to the relevant receptor or ligand and to CD3 as T cell marker, CD19 as B cell marker and CD11c as marker for myeloid cells, in particular DC. The expression of receptors and ligands was consistently most pronounced in the lung (Fig. 3), as compared to DLN and spleen (not shown). With regards to the receptors, we found that CD27 was clearly expressed on the great majority of T cells in DLN, spleen and lung throughout primary and memory responses (Fig. 3A). OX40 was found on a minority of T cells, which were most abundant in the lung during the primary response (Fig. 3A). 4-1BB, however, was virtually undetectable on T cells, but quite prominent on CD11c\textsuperscript{+} cells in lung (Fig. 3A), DLN and spleen (not shown). CD27 and OX40 were also present on CD11c\textsuperscript{+} cells in these organs, but to a lesser extent than 4-1BB (Fig. 3A and results not shown). Expression of the receptors on B cells was generally of low intensity and frequency, although 4-1BB was clearly detectable on B cells in the lung during the primary response (Fig. 3A).

The ligands of the TNF family are notoriously difficult to detect because they are transiently expressed, contingent upon immune activation. We found CD70, OX40L and 4-1BBL on T cells in DLN, spleen and lung, but at low frequency. The ligands were most prominent on CD11c\textsuperscript{+} cells, while they were also found on B cells (Fig. 3B). The primary

**Figure 3. Expression of receptors and ligands in lung during primary and secondary infection.**

At the indicated time points after primary or secondary infection, cells extracted from lungs were stained with antibodies for CD3, CD11c or CD19, combined with antibodies for CD27, OX40, 4-1BB, CD70, OX40 or 4-1BBL and analyzed by flow cytometry. (A) Percentage of lung-infiltrating CD3\textsuperscript{+} cells (T cells), CD11c\textsuperscript{+} cells (enriched for DC) and CD19\textsuperscript{+} cells (B cells) expressing CD27, OX40 or 4-1BB. (B) Percentage of lung-infiltrating CD3\textsuperscript{+} cells (T cells), CD11c\textsuperscript{+} cells (enriched for DC) and CD19\textsuperscript{+} cells (B cells) expressing CD70, OX40L, or 4-1BBL. (C) Flow cytometry dot plots showing fluorescence intensity of CD70, OX40L and 4-BBL staining on lung-infiltrating CD11c\textsuperscript{+} cells. In the upper right quadrant the percentage CD11c\textsuperscript{+} cells expressing the ligands is indicated.
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![Graphs showing the expression of CD3*, CD11c*, and CD19* on CD8+ T cells over days 3 to 14 for primary and secondary responses with various cytokines: CD27, OX40, 4-1BB, CD70, OX40L, and 4-1BBL.](image-url)

**Legend:**
- CD27
- OX40
- 4-1BB
- CD70
- OX40L
- 4-1BBL

**Day 3:**
- CD70: 0.48%
- OX40L: 1.80%
- 4-1BBL: 2.21%

**Day 6:**
- CD70: 1.19%
- OX40L: 7.96%
- 4-1BBL: 10.63%

**Day 8:**
- CD70: 0.23%
- OX40L: 1.35%
- 4-1BBL: 1.90%

**Day 10:**
- CD70: 0.04%
- OX40L: 0.38%
- 4-1BBL: 1.11%

**Day 14:**
- CD70: 0.03%
- OX40L: 0.07%
- 4-1BBL: 0.34%
data in Fig. 3C highlight the transient and activation-specific nature of CD70, OX40L and 4-1BB expression on CD11c+ cells in the lung, the site where they were most abundant. While OX40L and 4-1BB reached relatively high levels of cell surface expression, CD70 expression was very low, even at the peak of the response. Therefore, the percentages of CD70+ cells in figure 3B are probably an underestimate. From comparing expression of CD27, OX40 and 4-1BB and their ligands in the lung throughout primary and secondary responses, it appears that OX40/OX40L and 4-1BB/4-1BBL are much less prominently expressed in the secondary response (Fig. 3A,B).

These data indicate that receptor/ligand communication might take place at the site of priming as well as at the site of infection. CD11c+ cells (possibly DC) may be very important for presenting the ligands to their receptors on T cells at both sites. B cells, in particular at the effector site, may play a similar role. Receptor/ligand interaction may play a role during communication amongst primed T cells as well. Moreover, receptor/ligand interactions taking place during communication between CD11c+ cells, in particular 4-1BB/4-1BBL interactions, may modulate the T cell response indirectly. Finally, expression patterns suggest a more important role for OX40 and 4-1BB receptor/ligand interaction during the primary than during the secondary response.

**Formation of memory CD8+ T cells in the absence of CD27, OX40L or 4-1BBL**

The number of memory T cells formed after primary infection is an important parameter for the memory response. To study the effect of CD27-, OX40L- or 4-1BBL deficiency on the generation memory CD8+ T cells, the numbers of CD8+ T cells specific for the H2-D\(^b\)/NP\(_{366-374}\) complex were enumerated 6 weeks after primary infection. In blood, the percentage of T cells within the CD8+ population that were stained with H2-D\(^b\)/NP\(_{366-374}\) tetramers was more than 7-fold higher in infected mice than in naïve mice, indicating the presence of memory (Fig. 4A). Interestingly, in mice deficient for CD27, OX40L, or 4-1BBL, CD8+ memory T cell levels in blood were almost 2-fold reduced as compared to wild-type levels (Fig. 4A). In spleens of wild-type memory mice, numbers of T cells staining with H2-D\(^b\)/NP\(_{366-374}\) tetramers were increased about 6-fold as compared to numbers found in naïve mice. In CD27-/-, 4-1BBL-/- and OX40L-/- mice, levels of H2-D\(^b\)/NP\(_{366-374}\)-specific memory T cells were reduced about 2-fold as compared to those in wild-type mice (Fig. 4B). Interestingly, in the lungs of previously infected mice, numbers of T cells staining with H2-D\(^b\)/NP\(_{366-374}\) tetramers were about 10-fold higher than in naïve mice. These most likely represent at least in part tissue memory cells, since their adoptive transfer gave rise to a memory response (results not shown). As in the spleen, all three receptor/ligand systems promoted memory T cell formation in the lung, with CD27/CD70 and 4-1BB/4-1BBL having a greater effect than OX40/OX40L (Fig 4B).
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Figure 4. Formation of CD8+ T-cell memory in mice deficient for CD27, OX40L, or 4-1BBL.

(A) Blood was collected from wild-type (WT), CD27−/−, OX40L−/−, 4-1BBL−/−, mice at 6 weeks after infection, as well as from uninfected naïve wild-type mice (6 mice per group, pooled). Samples were stained with anti-CD8 mAb and H-2D^b/NP_366-374 tetramers and analyzed by flow cytometry. Bars represent percentage of tetramer positive cells within the CD8+ population. (B) Cells were harvested from spleens and lungs of wild-type (WT), CD27−/−, OX40L−/−, and 4-1BBL−/− mice at 6 weeks after influenza infection (4 mice per group). Samples were stained and analyzed as for (A). Bars represent mean number of virus-specific CD8+ T cells. Statistical analysis was performed as for Fig.1. Results are representative of two experiments.

We conclude that after intranasal influenza virus infection, generation of the CD8+ central and tissue memory T cell pools relies on the collective contributions made by CD27, OX40, 4-1BB and their ligands. Although OX40L-deficiency did not affect the accumulation of virus-specific effector CD8+ T cells in the primary response, it did reduce memory T-cell formation. This suggests that OX40/OX40L interactions promote T-cell survival in the contraction and/or memory phase of the T-cell response. Interaction between CD27, 4-1BB and their ligands may similarly do so and may in addition promote memory CD8+ T cell formation by incrementing the size of the CD8+ effector T cell pool.

Secondary expansion of memory T cells from CD27+/−, OX40L−/− or 4-1BBL−/− mice
To determine the contribution of CD27, OX40L and 4-1BBL to expansion and accumulation of memory CD8+ T cells during the secondary response, we performed adoptive transfer experiments. T cells were purified from spleens of wild-type, CD27+/−, OX40L−/− and 4-1BBL−/− mice, which had been infected with influenza virus 6 weeks earlier. These populations were stained with H-2D^b/NP_366-374 tetramers and the number of T cells used for adoptive transfer was adjusted so that each recipient mouse received an equal
number of H-2D\textsuperscript{b}/NP\textsubscript{366-374} T cells (see Fig. 4 for numbers of memory cells in this experiment). In this way, we corrected for the effects of CD27-, OX40L- and 4-1BBL-deficiency on memory T cell formation. Donor T cells were labeled with carboxyfluorescein diacetate ester (CFSE) and injected intravenously into wild-type recipient mice.

**Figure 5.** Secondary expansion of CD8\textsuperscript{+} memory T cells from CD27\textsuperscript{-}, OX40L\textsuperscript{-} or 4-1BBL\textsuperscript{-} mice in wild-type recipients.

Wild-type (WT), CD27\textsuperscript{-}, OX40L\textsuperscript{-} and 4-1BBL\textsuperscript{-} mice of the CD45.2 allotype were infected with influenza virus. At 6 weeks after primary infection, T cells were purified from spleen and stained with H-2D\textsuperscript{b}/NP\textsubscript{366-374} tetramers and anti-CD8 mAb and labeled with CFSE. T-cell samples, standardized to contain equal numbers of H-2D\textsuperscript{b}/NP\textsubscript{366-374}-specific T cells were adoptively transferred to wild-type mice of the CD45.1 allotype, which had been immunized 6 weeks earlier. Recipient mice were reinfected 2 days after adoptive transfer. At day 6 after infection, spleens, lungs and DLN were harvested and stained with anti-CD45.1 mAb, NP\textsubscript{366-374}/H-2D\textsuperscript{b} tetramers and anti-CD8 mAb and analyzed by flow cytometry. (A) Representative dot plots of WT spleen samples show gating on donor cells (CD45.1 negative) and analysis of donor T cells that have responded to virus infection (CFSE negative) by tetramer or anti-CD8 staining. (B) Absolute numbers of either tetramer\textsuperscript{+} or CD8\textsuperscript{+} responder T cells derived from the donor were calculated from the percentage tetramer\textsuperscript{+}/CD45.1\textsuperscript{+}/CFSE\textsuperscript{-} or CD8\textsuperscript{+}/CD45.1\textsuperscript{+}/CFSE\textsuperscript{-} cells and the total number of cells per organ. Bars represent mean values from 4 mice per time point. Statistical analysis was performed as for Fig. 1. The experiment shown is representative of two.
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Recipients had been infected 6 weeks earlier to create a memory environment. They were re-infected 2 days after adoptive transfer and analyzed 6 days after infection. Donor and recipient cells were discriminated based on expression of the CD45.2 and CD45.1 allotype, respectively. Virus-specificity was based on staining with H-2D\(^b\)/NP\(_{366-374}\) tetramer and responsiveness on loss of CFSE staining (Fig. 5A).

In lung, spleen and DLN, accumulation of H-2D\(^b\)/NP\(_{366-374}\)-specific T cells derived from primed CD27\(^-\), OX40L\(^-\) or 4-1BBL\(^-\) mice was significantly reduced as compared to accumulation of T cells from primed wild-type mice (Fig. 5B). The total pool of responding CD8\(^+\) T cells (based on CFSE loss) was also significantly decreased in case memory T cells were derived from CD27\(^-\) OX40L\(^-\) or 4-1BBL-deficient mice. This was particularly clear in lung, but could also be observed in the spleen. In DLN, only OX40L deficiency had a significant effect on the total CD8\(^+\) responder pool. We conclude that memory CD8\(^+\) T cells specific for the H2-D\(^b\)/NP\(_{366-374}\) complex, as well as memory CD8\(^+\) T cells with other virus specificities are dependent on the collective contributions of CD27, OX40L and 4-1BBL for their secondary expansion and establishment at the site of infection.

Support by OX40L and 4-1BBL on non-T cells is not required during the secondary response

In the experiment described above, memory T cells derived from OX40L\(^-\) or 4-1BBL\(^-\) mice normally expressed OX40 or 4-1BB, while their ligands were present in the wild-type recipients. We considered therefore that absence of OX40L or 4-1BB1 during the primary response might have compromised the intrinsic capacity of memory T cells from OX40L\(^-\) and 4-1BBL\(^-\) mice to expand upon renewed challenge. Subsequent experiments were designed to address this possibility.

To test whether OX40L or 4-1BBL were required throughout the secondary response, we determined the capacity of wild-type memory cells to expand upon renewed challenge in a ligand-deficient environment. Wild-type mice were infected with influenza virus to generate memory T cells. Six weeks later, T cells were purified from the spleens of these mice, labeled with CFSE and adoptively transferred to previously infected wild-type, OX40L\(^-\) or 4-1BBL\(^-\) mice. These recipients were re-infected with influenza virus 2 days later and analyzed 5 days after infection. Donor and recipient cells were discriminated based on CD45.1 and CD45.2 expression. Responding CD8\(^+\) T cells were characterized by CFSE dilution and H-2D\(^b\)/NP\(_{366-374}\) tetramer staining.

After influenza virus infection, wild-type memory CD8\(^+\) T cells transferred into OX40L\(^-\) or 4-1BBL\(^-\) recipients expanded and accumulated in spleen, lung and DLN to a similar extent as wild-type memory CD8\(^+\) T cells transferred into wild-type recipients (Fig. 6). Both H-2D\(^b\)/NP\(_{366-374}\) specific and total CD8\(^+\) memory T cells were independent of OX40L or 4-1BBL for their accumulation during the secondary response. In this system,
the only cells that may express OX40L or 4-1BBL throughout secondary T cell expansion are the transferred T cells themselves. We can conclude therefore, that memory CD8\(^+\) T cells do not require expression of OX40L or 4-1BBL on non-T cells throughout the secondary response for expansion and accumulation at the effector site.

**Figure 6.** Secondary expansion of wild-type memory CD8\(^+\) T cells in OX40L\(^-\) or 4-1BBL\(^-\) recipients. Wild-type mice of the CD45.1 allotype were infected with influenza virus. Six weeks later, T cells were isolated from spleen, labeled with CFSE and injected into wild-type (WT), OX40L\(^-\) or 4-1BBL\(^-\) recipients of the CD45.2 allotype that were infected 2 days after transfer. At day 5 after infection, cells from spleens, lungs and DLN were stained with anti-CD45.1 mAb and H-2D\(^b\)/NP\(_{366-374}\) tetramers or anti-CD8 mAb and analyzed by flow cytometry. Absolute numbers of either tetramer\(^+\) or CD8\(^+\) responder T cells were calculated from the percentage tetramer\(^+\)/CD45.1\(^+\)/CFSE\(^-\) or CD8\(^+\)/CD45.1\(^+\)/CFSE\(^-\) cells and the total number of cells per organ. Bars represent mean values from 3 mice per time point. Statistical analysis was performed as for Fig. 1. The experiment shown is representative of two.

**OX40L and 4-1BBL on non-T cells imprint the capacity for secondary expansion in CD8\(^+\) T cells during the primary response**

The finding that wild-type memory T cells could expand well in OX40L\(^-\) or 4-1BBL\(^-\) recipients suggested that either ligands on the memory T cells themselves supported secondary expansion, or that the potential for secondary expansion was imprinted into T cells by receptor/ligand interactions that occurred during the primary response. To address these possibilities, we primed wild-type T cells in OX40L\(^-\) or 4-1BBL\(^-\) recipients, monitored their conversion to CD8\(^+\) memory T cells and subsequently let standardized numbers of memory cells respond to re-challenge in wild-type mice. In this experiment, we also tested the capacity of CD8\(^+\) T cells from the OX40L\(^-\) and 4-1BBL\(^-\) recipients to form memory and to respond to re-challenge in wild-type mice.
This set-up also allowed us in the first place to determine the capacity wild-type CD8+ T cells to form memory in an OX40L−/− or 4-1BBL−/− recipients. This capacity (as monitored in blood) was reduced as compared to the wild-type situation (Fig. 7B). In fact, wild-type CD8+ T cells were as inadequate as OX40L−/− or 4-1BBL−/− CD8+ T cells to form H-2Db/NP366-374 specific memory in OX40L−/− or 4-1BBL−/− recipients. Apparently, CD8+ T cells require stimulation of OX40 and 4-1BB by OX40L and 4-1BB on non-T cells for memory formation.

Secondary responsiveness of adoptively transferred memory cells, standardized for numbers of H-2Db/NP366-374-specific T cells, was read out by challenging the secondary recipients with influenza virus 2 days after transfer. Six days after infection, spleens, lungs and DLN were harvested and absolute numbers of H-2Db/NP366-374-specific T cells as well as total CD8+ T cells from the donor mice were determined. As control served wild-type T cells that had been primed in wild-type mice (Fig. 7A). As also shown in Fig. 5, secondary expansion of H-2Db/NP366-374-specific memory T cells from primed OX40L−/− or 4-1BBL−/− mice in wild-type recipients was impaired, as evident from their significantly decreased accumulation in spleen. Accumulation of H-2Db/NP366-374-specific effector T cells in the lung was also impaired (Fig. 7C). Although less pronounced, these defects were also apparent when total CD8+ T cells from primed OX40L−/− or 4-1BBL−/− donors were analyzed (Fig. 7C). Interestingly, wild-type CD8+ memory T cells primed in an OX40L−/− or 4-1BBL−/− environment were similarly impaired in their capacity for secondary responsiveness in wild-type recipients as were memory CD8+ T cells from primed OX40L−/− or 4-1BBL−/− mice. This was evident from the reduced expansion of H-2Db/NP366-374-specific T cells in the spleen and their reduced accumulation in the lung.

The defect in the lung was also statistically significant for the total CD8+ T cell population (Fig. 7C). From this experiment, we derive the conclusion that triggering of OX40 or 4-1BB by their ligands on non-T cells during the primary response imprints into memory CD8+ T cells the potential to efficiently expand and accumulate upon for secondary challenge.

**Discussion**

In this study, we have determined that OX40, 4-1BB and CD27 collectively shape CD8+ T cell memory, both in terms of numbers of memory T cells formed and their capacity to accumulate upon secondary challenge. The contributions of CD27-, OX40- and 4-1BB receptor/ligand systems to T cell responsiveness had not previously been compared side-by-side in the same model of antigenic challenge. Unexpectedly, we have also revealed that OX40 and 4-1BB endow memory CD8+ T cells during their formation with an improved capacity for secondary responsiveness. Whether CD27 can do the same has not been investigated here, since CD70-deficient mice are not available.
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A

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Isolated T cells 2nd recipient (primed)

B

Donor: WT (CD45.1)
Recipient: WT

Recipient memory, WT
Donor memory, WT

WT in WT

WT in OX40L-

WT in 4-1BBL-

C

Blood

Spleen

Lung

DLN
OX40, 4-1BB and CD27 shape CD8+ T cell memory

Figure 7. Wild-type T cells primed in absence of OX40L or 4-1BBL show defective secondary expansion.
(A) Schematic presentation of the experimental design. Purified splenic T cells from naïve wild-type (WT) CD45.1+ mice were injected into CD45.2+ wild-type, OX40L" or 4-1BBL" mice, which were infected 2 days later. After 6 weeks, T cells were purified from spleen and stained with H-2D\(^{b}\)/NP\(_{366-374}\) tetramers as well as with CD45.1-specific mAb to determine memory T cell formation (see panel B). Next, populations of purified splenic (memory) T cells from the first recipients, standardized to contain 0.5.10\(^5\) H-2D\(^{b}\)/NP\(_{366-374}\)-specific T cells, were injected into primed wild-type recipients. CD45.1+ T cells from wild-type donors were injected into CD45.2+ second recipients and CD45.2+ T cells from OX40L" or 4-1BBL" first recipients into CD45.1+ second recipients. (B) Dot plots show CD45.1+ donor-derived and CD45.1+ (CD45.2+) recipient-derived memory T cell populations with H-2D\(^{b}\)/NP\(_{366-374}\) specificity in blood, as detected 6 weeks after infection of the first recipients. Bars indicate percentage of H-2D\(^{b}\)/NP\(_{366-374}\) cells of these populations. Data are derived from pooled samples of 3 mice per group. (C) Second recipients were re-infected 2 days after transfer. At day 5 after infection, cells from spleens, lungs and DLN were stained with anti-CD45.1 mAb and H2-D\(^{b}\)/NP\(_{366-374}\) tetramers and anti-CD8 mAb and analyzed by flow cytometry. Absolute numbers of either H-2D\(^{b}\)/NP\(_{366-374}\) or CD8+ T cells were calculated from the percentage tetramer+/CD45.1+ or CD8+/CD45.1+ cells and the total number of cells per organ. Bars represent mean values from 3 mice per time point. Statistical analysis was performed as for Fig. 1. The experiment shown is representative of two.

As shown for OX40 receptor deficiency (Kopf et al., 1999), we found that OX40L deficiency did not compromise the primary CD8+ effector T cell response to intranasally delivered influenza virus. OX40-Ig fusion protein was recently shown to reduce both CD4+ and CD8+ T-cell numbers in the lungs of mice primed with HKx31 influenza virus, which is the first in vivo evidence that OX40 can promote primary CD8+ T cell responses (Humphreys et al., 2003). In a model of intraperitoneal injection with influenza virus (HKx31), 4-1BBL-deficiency did not affect CD8+ effector T-cell accumulation in the spleen (Bertram et al., 2002). In our model of intranasal virus delivery, however, 4-1BBL-deficiency did reduce generation of the CD8+ effector T cell pool in DLN and its establishment in the lung. Consistent with Betram et al. (2002), we found no contribution of 4-1BB/4-1BBL to the primary T cell response in the spleen. CD27 similarly does not affect the primary CD8+ T cell response in this organ (Hendriks et al., 2003). We believe that the splenic microenvironment may be different from DLN and lung tissue in that it offers alternative modes of survival support to activated T cells.

We have found that CD27 makes a greater contribution to the primary CD8+ T cell response than 4-1BB/4-1BBL. This can be explained by the fact that CD27 is already expressed on naïve T cells and contributes to T cell survival from the moment of priming. Nevertheless, CD27 and 4-1BBL make complementary contributions to the primary response. Possibly, their mechanism of action is the same, but activated T cells may meet CD70 and 4-1BBL at different moments after their initial activation. Alternatively, CD27 and 4-1BB may upregulate different inhibitory Bcl-2 family members, which differ in their capacity to counteract certain pro-apoptotic signals that operate in activated T cells. A third
The possibility is that there is a more profound distinction between CD27 and 4-1BB in their mechanism of action.

With regards to the formation of memory T cells, it is well-established that deliberate ligation of OX40 or 4-1BB prevents clonal deletion of previously activated T cells and enlarges the memory T cell pool in the spleen. In case of OX40, this effect was more profound for CD4⁺ T cells than for CD8⁺ T cells, while in case of 4-1BB it was the other way around (Maxwell et al., 2000; Takahashi et al., 1999). Steady state memory T cell levels have been documented in 4-1BBL−/− mice after intraperitoneal challenge with influenza virus (Betram et al. 2002). In that model, virus-specific CD8⁺ T cells were about two fold reduced at day 38 after primary infection. We here corroborate the requirement for 4-1BB/4-1BBL in CD8⁺ memory formation. In addition, we find that CD27/CD70 and OX40/OX40L shape CD8⁺ T cell memory. In the influenza model absolute numbers of H2Dᵇ/NP₃₆₆-₃₇₄ memory T cells are low, but tetramer staining is manifold over background levels found in naïve mice. Moreover, adoptive transfer experiments for spleen and lung corroborated the existence of memory on basis of kinetics and efficiency of the secondary response (our unpublished results). The contributions of CD27, CD70 and OX40L to CD8⁺ T cell memory in the circulation were comparable in magnitude. Our data indicate that CD27, OX40L and 4-1BBL also contribute to tissue memory formation of CD8⁺ T cells. Interesting in this respect is that expression of CD70, OX40L and 4-1BBL is most pronounced at the effector site. Unexpectedly, both DC and B cells in the lung carried these ligands. We suggest that communication between effector T cells and antigen presenting cells (DC, B cells) via these receptor/ligand systems at the tissue site may regulate the size of the effector T cell pool and the extent of effector T cell contraction. The finding that OX40L deficiency did not affect the size of the effector T cell pool, but compromised the size of the memory T cell pool, suggests that it acted during the contraction and/or memory phase. Complementarity between the three receptor/ligand systems seems to lie in part in the timing of their involvement in the primary response. In this scenario, CD27 would be first and OX40 the last to make a pro-survival contribution.

We had difficulty to detect OX40 and 4-1BB on T cells in vivo, but in vitro studies have proven that these receptors directly transmit survival signals into T cells (Rogers et al., 2001; Lee et al., 2002). Therefore, we assume that the effects on T cell responsiveness observed in OX40L− and 4-1BBL-deficient mice are at least in part due to defective signaling via OX40 and 4-1BB into T cells. However, the detection of both receptors and ligands on CD11c⁺ cells in infected mice has warned us that effects on T cells may also in part be indirect, i.e. proceed via the modulation of DC function. Our novel finding that CD11c⁺ cells can express both CD70 and CD27 indicates that these may similarly effect T cell function indirectly.
OX40, 4-1BB and CD27 shape CD8+ T cell memory

An important subject of recent studies is the question whether T cell responses proceed according to a pre-established program after initial antigen encounter. TCR stimulation triggers a developmental program in naïve CD8+ T cells, allowing them, in the subsequent absence of antigen, to divide at least seven times, to develop cytolytic effector functions and to acquire memory characteristics (Van Stipdonk et al., 2001; Keach and Ahmed, 2001). However, our data argue that antigen is an important factor in controlling T-cell survival and the extent of memory formation. We postulate that when antigen wanes, CD70, OX40L and 4-1BBL disappear and with them the pro-survival effects of their receptors. In such a scenario, antigen does not necessarily control effector and memory cell differentiation, but it does determine the amount of effector and memory cells formed. Presumably, the requirement for continuous input via CD27 and its related receptors into T cells is required to maintain expression of inhibitory Bcl-2 family members. In the memory phase, pro-survival support comes from cytokines such as IL-15, which can also upregulate inhibitory Bcl-2 family members (Schluns and Lefrancois, 2003).

Evidence has been presented recently that CD4+ T cells can program the capacity for secondary expansion into CD8+ T cells during priming (Janssen et al., 2003; Shedlock et al., 2003; Sun et al., 2003). Whether the effect of OX40 and 4-1BB triggering on memory formation and secondary responsiveness impacts directly on CD8+ T cells or affects these indirectly via CD4+ T cells remains to be shown. However, it is excluded that OX40L or 4-1BBL on CD4 T cells regulate CD8+ memory formation and responsiveness, since we have shown that ligands on non-T cells are important for this. We do not know when the programming for secondary expansion occurs, or what it entails at a molecular level. Since memory T cells are slowly cycling (Schluns and Lefrancois, 2003), it must be a capacity that can be transmitted to the daughter cells and therefore can truly be termed "programming". A recent paper by Bertram et al. (2004) seems to contradict our findings, since secondary expansion of primed T cells from 4-1BBL−/− mice in wild-type recipients was not impaired. We believe that the difference may be due to the intraperitoneal challenge with influenza virus that was used. This may involve a mode of priming (in the spleen) that bypasses the need for survival support by 4-1BB and its relatives. Our collective data strongly support the idea that the deliberate offering of CD70, OX40L and 4-1BBL during priming may be a potent strategy to achieve potent and long-lasting immunity.

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Experimental Procedures

Mice

Mice were bred in the facility of The Netherlands Cancer Institute under pathogen-free conditions and used for experiments at 6 to 12 weeks of age. Animal experiments were carried out according to institutional and national guidelines. CD27⁺, OX40L⁻ and 4-1BBL⁻ mice were generated and phenotyped by PCR as described (Hendriks et al., 2000; Murata et al., 2000; DeBenedette et al., 1999). They were backcrossed for multiple generations to a C57BL/6 background. Mice were of the CD45.2 allotype, unless specified otherwise.

Flow cytometry

Lungs, spleens and DLN were forced through a nylon mesh in Iscove’s Modified Dulbecco’s Medium (IMDM) with 8% FCS to acquire single cell suspensions. Erythrocytes were lysed on ice for 2 min in 0.14 M NH₄Cl, 0.017 M Tris-HCl, pH 7.2. Cells were pre-incubated with Fc Block (mAb to CD16/CD32, 2.4G2, BD Biosciences) and washed in staining buffer (PBS, 0.5% bovine serum albumin (BSA), 0.01% sodium azide). Next, cells were incubated with specific antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC). APC-labeled tetramers of the murine MHC class I H2-Dᵇ heavy chain, β2 microglobulin and the influenza NP366-374 peptide ASNENMDAM were prepared as described (Haanen et al., 1999). After incubation with respective antibodies and tetramers, cells were washed and analyzed using a FACSCalibur and CELLQuest software (Becton Dickinson, Mountain View, CA). Propidium iodide was added just before analysis to stain dead cells, which were excluded from further analysis. Monoclonal antibodies (mAbs) used for immunofluorescence were anti-CD3e, 500A2; anti-CD8b.2, 53-5.8; anti-CD4, GK1.5; anti-CD11c, N-418; anti-CD27, LG.3A10; anti-CD45R/B220, RA3-6B2; anti-CD45.1, A20; anti-OX40, OX-86; anti-4-1BB, 1AH2 (subclone of 53A2); anti-CD70, 3B9; anti-OX40L, RM134L; anti-4-1BBL, TKS-1. Antibodies were purchased from BD Biosciences or derived from available hybridoma cells.

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, The Netherlands. Mice were anesthetized and infected intranasally with 50 μl Hank’s balanced salt solution containing 25 hemagglutinin units of virus to induce primary responses. Six weeks later, 100 hemagglutinin units of the same virus were used to induce memory responses. In this model of viral infection of C57BL/6 mice, we can only read out the CD8⁺ T-cell response with the aid of MHC tetramers, since the immunodominant epitope for CD4⁺ T cells is undefined.

Preparation of purified T cells

Cells suspensions were prepared as described above, passed over nylon wool (Polysciences, Warrington, MA) and incubated on ice for 30 min with mAb M5/114.15.2 to MHC class II (BD Biosciences). This was followed by 30 min of incubation on ice with 100 μl goat anti-mouse Ig-
coated magnetic beads and 20 μl sheep anti-rat Ig-coated magnetic beads (Advanced Magnetics Inc.) per 10^7 cells. Beads were removed by magnetic sorting.

Adoptive transfers
For all adoptive transfers purified splenic T cells were used. Where indicated, donor T cells were labeled with CFSE before adoptive transfer by incubation at a concentration of 5 x 10^7 cells/ml in PBS, containing 0.1% BSA and 5 μM CFSE, for 10 min at 37°C. Labeling was quenched with 10 ml of cold medium with 10% FCS and cells were washed twice with IMDM with 8% FCS prior to use. Donor T cells were suspended in 200 μl Hank’s balanced salt solution and injected into the tail vein of recipient mice at the indicated concentrations and mice were infected 2 days later. Recipient mice were used for adoptive transfer 6 weeks after primary virus infection to ensure a primed environment for transferred T cells. Organs were taken from recipient mice for analysis by flow cytometry at day 5 or 6 after infection, as indicated. For the experiment depicted in Fig. 5, purified T cells isolated from 4 primed CD45.2^+ wild-type, CD27^-/^-OX40L^-/^- and 4-1BBL^-/^- mice were pooled and stained with H2-D^b/NP_366-374 tetramers. T cell populations used for adoptive transfer were standardized to contain 1 x 10^5 H2-D^b/NP_366-374-specific T cells per CD45.1^+ wild-type recipient mouse (n=4). For the experiment depicted in Fig. 6, donor T cells were derived from 3 primed CD45.1^+ wild-type mice, pooled and injected at 10 x 10^6 per mouse into CD45.2^+ recipient mice of wild-type, OX40L^-/^- or 4-1BBL^-/^- phenotype (n=3 per group). For the experiment depicted in Fig. 7, 25 x 10^6 purified T cells from four naïve CD45.1^+ mice were first injected into CD45.2^+ wild-type, OX40L^-/^- or 4-1BBL^-/^- mice, which were infected 2 days later (n=3 per group). After 6 weeks, T cells were purified from spleen, pooled and stained with H2-D^b/NP_366-374 tetramers as well as with CD45.2-specific mAb. Populations of purified T cells standardized to contain 0.5 x 10^5 H-2D^b/NP_366-374-specific T cells of CD45.1^+ or CD45.2^+ phenotype were injected per wild-type recipient mouse of the CD45.2^+ or CD45.1^+ phenotype, respectively (n=3 per group).

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