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During Viral Infection of the Respiratory Tract, CD27, 4-1BB, and OX40 Collectively Determine Formation of CD8⁺ Memory T Cells and Their Capacity for Secondary Expansion¹

Jenny Hendriks,* Yanling Xiao,* John W. A. Rossen,[†] Koenraad F. van der Sluijs,[‡] Kazuo Sugamura,[§] Naoto Ishii,[§] and Jannie Borst^{2*}

Independent studies have shown that CD27, 4-1BB, and OX40 can all promote survival of activated CD8⁺ T cells. We have therefore compared their impact on CD8⁺ memory T cell formation and responsiveness within one, physiologically relevant model system. Recombinant mice, selectively lacking input of one or two receptors, were challenged intranasally with influenza virus, and the immunodominant virus-specific CD8⁺ T cell response was quantified at priming and effector sites. Upon primary infection, CD27 and (to a lesser extent) 4-1BB made nonredundant contributions to accumulation of CD8⁺ virus-specific T cells in draining lymph nodes and lung, while OX40 had no effect. Interestingly though, in the memory response, accumulation of virus-specific CD8⁺ T cells in spleen and lung critically depended on all three receptor systems. This was explained by two observations: 1) CD27, 4-1BB, and OX40 were collectively responsible for generation of the same memory CD8⁺ T cell pool; 2) CD27, 4-1BB, and OX40 collectively determined the extent of secondary expansion, as shown by adoptive transfers with standardized numbers of memory cells. Surprisingly, wild-type CD8⁺ memory T cells expanded normally in primed OX40 ligand- or 4-1BB ligand-deficient mice. However, when wild-type memory cells were generated in OX40 ligand- or 4-1BB ligand-deficient mice, their secondary expansion was impaired. This provides the novel concept that stimulation of CD8⁺ T cells by OX40 and 4-1BB ligand during priming imprints into them the capacity for secondary expansion. Our data argue that ligand on dendritic cells and/or B cells may be critical for this. *The Journal of Immunology*, 2005, 175: 1665–1676.

An effective immune response relies on the clonal amplification of Ag-specific T cells and their accumulation at the site of infection. For long-term protection, part of the Ag-specific T cell pool must be retained as memory cells, which respond rapidly to renewed challenge. Naive 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 Ig superfamily members, including CD28 and ICOS, and TNFR family members, such as CD27, 4-1BB, and OX40 (1, 2). Like the TCR, CD28 and relatives signal via tyrosine kinases, while costimulatory TNFR family members signal via TNFR-associated factors (3). Both mechanisms activate the NF- κ B and Jun kinase pathways, posing the question as to 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 (4). Upon intranasal infection with

influenza virus, CD27 and CD28 equally contributed to expansion of virus-specific CD8⁺ T cells and their accumulation at the effector site. Unlike CD28, CD27 did not affect cell cycle entry or activity. Rather, CD27 protected primed T cells from apoptosis (4) and appeared to complement CD28 in its antiapoptotic effect (5). 4-1BB and OX40, the closest relatives of CD27 (1, 2), also promote activated T cell survival, apparently by stimulating expression of inhibitory Bcl-2 family members (6, 7). Although this suggests redundancy between CD27, 4-1BB, and OX40, complementarity may lie in their selective effects on certain cell populations and the timing of their involvement. The expression patterns of these receptors give a clue that this might be the case. CD27 is already expressed on naive T cells (8, 9), but 4-1BB and OX40 are induced by TCR signals, with CD28 enhancing their expression (6, 10–12). Accordingly, in activated T cells lacking 4-1BB or OX40 signaling, survival is not compromised initially, but it is defective in the later divisions (6, 13).

The ligands are TNF-related transmembrane molecules, which emerge transiently, under strict control of Ag receptors and TLRs (3, 8). CD70, the ligand of CD27, 4-1BB ligand (4-1BBL),³ and OX40 ligand (OX40L) have all been found on activated lymphocytes and mature dendritic cells (DC) (10, 14–17), indicating that receptor/ligand interactions come into play during communication between T cells and APCs as well as among effector T cells. Limited data are available on their expression in vivo. Deliberate constitutive expression of CD70, 4-1BBL, or OX40L by transgenesis upsets lymphocyte homeostasis, leading to immunodeficiency or autoimmunity, indicating that the transient availability of these ligands is crucial to prevent pathogenesis (18–21).

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³ Abbreviations used in this paper: 4-1BBL, 4-1BB ligand; DC, dendritic cell; DLN, draining lymph node; NP, nuclear protein; OX40L, OX40 ligand.

Phenotypes of receptor- and ligand-deficient mice are subtle as compared with phenotypes of mice in which the receptors are deliberately stimulated. Agonistic anti-4-1BB or anti-OX40 Abs rescued T cells from activation-induced cell death and promoted memory formation (22, 23). In CD70 transgenic mice, effector CD4⁺ and CD8⁺ T cells develop in the absence of deliberate antigenic challenge (18, 19), and in nonimmunized OX40L transgenic mice, CD4⁺ effector T cells accumulate (20). In addition, in CD70 transgenic mice, CD8⁺ effector T cells, and in OX40L transgenic mice, CD4⁺ effector T cells showed decreased contraction after antigenic challenge (20, 24).

Like the studies with deliberate receptor triggering, initial analysis of receptor- and ligand-deficient mice suggested that 4-1BB and OX40 selectively impact on CD8⁺ and CD4⁺ effector T cells, respectively, while CD27 impacts on both. In CD27^{-/-} mice infected intranasally with influenza virus, accumulation of both CD4⁺ and CD8⁺ effector T cells was reduced (25). In mice lacking 4-1BBL, fewer CD8⁺ effector T cells and memory T cells developed upon i.p. challenge with influenza virus (26–28). OX40- and OX40L-deficient mice had reduced primary CD4⁺ T cell responses to intranasal virus and to protein Ags (29–31). Lower frequencies of effector CD4⁺ T cells were generated late in the primary response, and fewer CD4⁺ memory T cells developed (32). However, recent work indicates that this specialization is not absolute: OX40 can promote primary CD8 T cell responses (33, 34) by supporting CD8⁺ T cell survival (34).

There are many data on the role of costimulatory receptors in primary responses, but less is known about their importance for memory responses. In our model of intranasal delivery of influenza virus, accumulation of CD8⁺ effector T cells in the memory response critically depended on both CD27 and CD28 (4, 25). Upon i.p. delivery of influenza virus, 4-1BBL^{-/-} and OX40L^{-/-} mice were selectively impaired in CD8⁺ and CD4⁺ memory T cell responses, respectively (28, 35). In a model of virus-induced lung inflammation, OX40 also controlled accumulation of CD4⁺ effector cells in the memory response (33, 36). These data suggest that CD27 and 4-1BB are important for antiviral CD8⁺ memory responses, while OX40 is not.

In this study, we used recombinant mice deficient for CD27, 4-1BB, and/or OX40 signaling to address the question as to whether these receptors make complementary contributions to the same antiviral CD8⁺ T cell response. We discriminated between effects on the primary response, memory formation, and secondary expansion. We found, unexpectedly, that formation of the same CD8⁺ memory T cell pool, as well as its capacity for secondary expansion, depended on the collective, nonredundant contributions of CD27, 4-1BB, and OX40. In addition, we revealed that 4-1BB and OX40 endowed CD8⁺ T cells with the capacity for secondary expansion during the primary response.

Materials and Methods

Mice

Wild-type, CD27^{-/-} (25), 4-1BBL^{-/-} (27), and OX40L^{-/-} (31) mice were on C57BL/6 background and used for experiments at 6–10 wk of age, in accordance with institutional and national guidelines. Mice were of the CD45.2 allotype, unless specified otherwise.

Flow cytometry

Lungs, spleens, and lung draining lymph nodes (DLN)³ were forced through a nylon mesh in 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 preincubated with Fc block (mAb to CD16/CD32, 2.4G2; BD Biosciences) and washed in staining buffer (PBS, 0.5% BSA, 0.01% sodium azide). Next, cells were incubated with specific Abs conjugated to FITC, PE, or allophycocyanin. Allophycocyanin-labeled

tetramers of the murine MHC class I H-2D^b H chain, β_2 -microglobulin, and the influenza nuclear protein (NP)_{366–374} peptide ASNENMDAM were prepared as described and used in combination with anti-CD8 mAb (37). Cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences). Propidium iodide-stained dead cells were excluded from analysis. mAb used for immunofluorescence were anti-CD3 ϵ , 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; and anti-4-1BBL, TKS-1.

Virus infection

Influenza virus strain A/NT/60/68 was grown, purified, and tested for hemagglutinin activity and infectious titers in the Department of Virology, Erasmus University Rotterdam. Mice were anesthetized and infected intranasally with 50 μ l of HBSS 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, because the immunodominant epitope for CD4⁺ T cells is undefined.

Determination of virus-specific Ig levels and virus-neutralizing activity

Virus-specific Ig levels were determined, as described previously (38). In brief, blood was collected from the tail vein in heparin-treated tubes. Density gradient-purified influenza virus was coated onto 96-well flat-bottom microtiter plates (Nunc). Wells were incubated with serially diluted sera, followed by biotinylated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 mAb (Southern Biotechnology Associates) and streptavidin-conjugated HRP (Sigma-Aldrich). Substrate 3,3',5,5' tetramethylbenzidine (Merck) was used to develop the reaction. Endpoint titers were expressed as reciprocal log₃ of the last dilution, which gave an OD₄₅₀ of ≥ 0.1 OD unit above the OD₄₅₀ of the negative control (pooled serum from nonimmunized mice). Virus-neutralizing activity of serum Ig was tested with the hemagglutination inhibition assay. In this assay, serial serum dilutions are incubated with a standard dose of influenza virus and chicken RBC.

Determination of viral loads

Lungs were harvested at the indicated days after infection and homogenized at 4°C in sterile saline with a tissue homogenizer (BioSpec Products). Lung homogenate was dissolved in TRIzol reagent, and RNA was isolated according to the manufacturer's protocol (Invitrogen Life Technologies). cDNA synthesis was performed using a random hexamer cDNA synthesis kit (Applera). cDNA samples were assayed in duplicate in a 25- μ l reaction mixture containing 5 μ l of cDNA, 12.5 μ l of 2 \times Taqman Universal PCR Master Mix (Applied Biosystems), 900 nM influenza virus A forward primer (AAG ACC AAT CCT GTC ACC TCT GA), 900 nM influenza virus A reverse primer (CAA AGC GTC TAC GCT GCA GTC C), and 200 nM influenza A probe (TTT GTG TTC ACG CTC ACC GT) (39). The fluorogenic probe was labeled with a 5' reporter dye, FAM and with a 3' quencher dye, TAMRA. Amplification and detection were performed in the ABI Prism 7700 sequence-detection system (Applied Biosystems), using the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The viral load present in a sample was calculated using a standard curve of influenza virus in every assay run. Differences between groups were analyzed by a Mann-Whitney U test using GraphPad Prism version 4.00 for Windows (GraphPad).

Preparation of purified T cells

Cell suspensions were passed over nylon wool (Polysciences) and incubated on ice for 30 min with mAb M5/114.15.2 to MHC class II (BD Biosciences), followed by 30 min of incubation on ice with 100 μ l of goat anti-mouse Ig-coated magnetic beads and 20 μ l of sheep anti-rat Ig-coated magnetic beads (Advanced Magnetics) per 10⁷ 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 (Molecular Probes) before adoptive transfer (4). Donor T cells were suspended in 200 μ l of HBSS and injected into the tail vein of recipient mice at the indicated concentrations, and mice were infected 2 days later. Recipient mice had been infected 6 wk earlier to ensure a primed environment for transferred T cells. For the experiment depicted in Fig. 5, purified T cells isolated from four primed

CD45.2⁺ wild-type, CD27^{-/-}, OX40L^{-/-}, and 4-1BBL^{-/-} mice were pooled and stained with H-2D^b/NP₃₆₆₋₃₇₄ tetramers. T cell populations used for adoptive transfer were standardized to contain 1×10^5 H-2D^b/NP₃₆₆₋₃₇₄-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 three primed CD45.1⁺ wild-type mice, pooled, and injected at 10×10^6 per mouse into CD45.2⁺ recipient mice of wild-type, OX40L^{-/-}, or 4-1BBL^{-/-} phenotype ($n = 3$). For the experiment depicted in Fig. 7, 25×10^6 purified T cells from four naive CD45.1⁺ mice were first injected into CD45.2⁺ wild-type, OX40L^{-/-}, or 4-1BBL^{-/-} mice, which were infected 2 days later ($n = 3$). After 6 wk, T cells were purified from spleen, pooled, and stained with H-2D^b/NP₃₆₆₋₃₇₄ tetramers as well as with CD45.2-specific mAb. Populations of purified T cells standardized to contain 0.5×10^5 H-2D^b/NP₃₆₆₋₃₇₄-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 ($n = 3$).

Results

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

Intranasal challenge with influenza virus A/NT/60/68 results in T cell priming in mediastinal lung DLN, followed by an influx of effector T cells into the lung (37). The infection with this strain is mild and not accompanied by extensive lung inflammation or formation of BALT. Among different influenza virus strains tested, A/NT/60/68 best reveals the CD27-deficient phenotype (our unpublished results). To follow virus-specific CD8⁺ T cells, we used MHC H-2D^b tetramers loaded with the immunodominant NP₃₆₆₋₃₇₄ peptide. In wild-type mice, H-2D^b/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. 1). In the lungs of CD27^{-/-} mice, accumulation of H-2D^b/NP₃₆₆₋₃₇₄-specific T cells was greatly reduced, as documented previously (4, 25). In absence of 4-1BBL, the CD8⁺ T cell response in lung was also

reduced, but to a lesser extent. Similar observations were made for generation of the virus-specific CD8⁺ T cell pool in DLN: both CD27 and 4-1BB made a contribution, with CD27 having the greatest effect. In contrast, OX40L deficiency did not affect the accumulation of CD8⁺ virus-specific T cells in either lung or DLN. In the spleen, none of the receptor/ligand systems made a significant contribution to the accumulation of virus-specific CD8⁺ T cells.

To assess a possible redundancy among CD27, 4-1BB, and/or OX40 in establishing the size of the virus-specific CD8⁺ T cell pool, we also analyzed responses in 4-1BBL/CD27^{-/-} and OX40L/CD27^{-/-} double-deficient mice (Fig. 1). In DLN and lung, additional 4-1BBL or OX40L deficiency did not exacerbate the phenotype of CD27-deficient mice. In addition, redundancy between CD27 and 4-1BB or OX40 did not explain why virus-specific CD8⁺ T cell accumulation in the spleen was hardly affected in single-deficient mice, because in double-deficient mice no statistically significant reductions in cell accumulation were observed either.

We conclude that upon primary intranasal infection with influenza virus, CD27 makes the most important contribution to generation of virus-specific CD8⁺ T cells in DLN and their accumulation at the site of infection. 4-1BB also contributes, but to a lesser extent, while OX40 does not. The function of CD27 is nonredundant with that of 4-1BB or OX40. In the spleen, primed CD8⁺ T cells appear not critically dependent on any of these receptor systems for their accumulation.

Ab responses are not affected in absence of CD27 and 4-1BBL or OX40L

We have shown previously that infection with A/NT60/68 influenza virus results in virus-specific IgG production, which reaches plateau levels between day 14 and 21 after challenge. These Ig levels persist long-term and are only marginally incremented upon renewed challenge with the same virus. The absence of CD27 did not affect Ig production, but only delayed germinal center formation (38). In mice, single deficient for CD27, 4-1BBL, or OX40L, IgM, IgG1, IgG2a, IgG2b, and IgG3 responses were unaltered as compared with responses in wild-type mice (results not shown). Even in mice double deficient for CD27 and 4-1BBL or CD27 and OX40L, these responses proceeded normally and plateau Ig levels attained were similar in all mouse strains (Fig. 2A). Also at the peak of the secondary T cell response, IgG titers were not significantly altered in double-deficient mice as compared with those in wild-type mice (Fig. 2A). We conclude that the contribution of CD27, 4-1BB, or OX40 is not required for virus-specific IgG production in this model system. Redundancy between CD27 and 4-1BB or OX40 does not explain the lack of effect.

Virus-neutralizing activity of serum Ig was assessed at day 21 after infection by hemagglutination inhibition assay and found to be comparable in wild-type, 4-1BBL/CD27^{-/-}, and OX40L/CD27^{-/-} mice. Viral load was determined by PCR in total lung extracts of wild-type, 4-1BBL/CD27^{-/-}, and OX40L/CD27^{-/-} mice at days 4, 7, 14, and 21 after primary infection (Fig. 2B). In agreement with the unaltered IgG response in the different recombinant strains and the lack of effect of CD27 and 4-1BBL or OX40L deletion on the virus-neutralizing activity of serum Ig, we found that clearing of influenza virus from the lungs of wild type and double deficient proceeded with similar kinetics and efficacy. Viral load peaked at or before day 4 after infection, and virus was cleared by day 21. The only statistically significant difference ($p < 0.05$) was for OX40L/CD27^{-/-} mice vs wild-type and 4-1BBL^{-/-} mice at day 7.

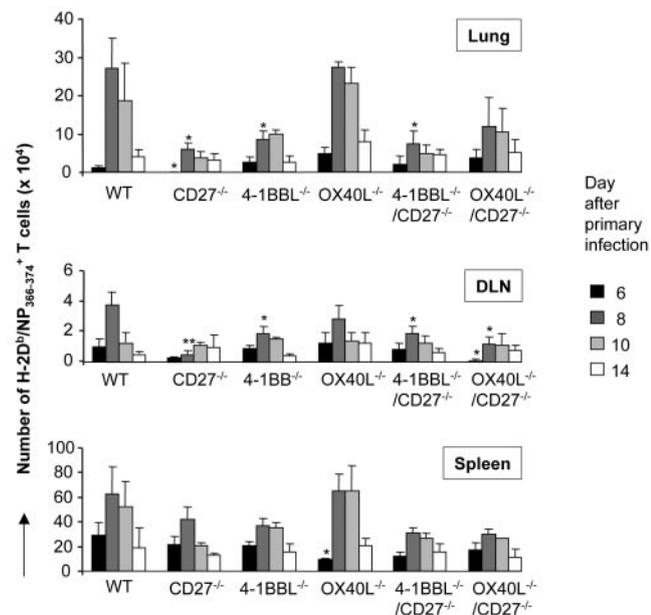


FIGURE 1. Primary CD8⁺ T cell responses in absence of CD27, 4-1BB, and/or OX40L signals. Wild-type (WT) and recombinant mice were infected with influenza virus. At the indicated days after infection, cells were isolated from lungs, DLN, and spleens; counted; stained with anti-CD8 mAb and H-2D^b/NP₃₆₆₋₃₇₄ tetramers; and analyzed by flow cytometry. Bars represent mean values ($n = 4$); error bars indicate SEM. Two-tailed Student's *t* test indicated significant differences compared with wild-type values for $p \leq 0.05$ (*) and $p \leq 0.01$ (**). The experiments are representative of two.

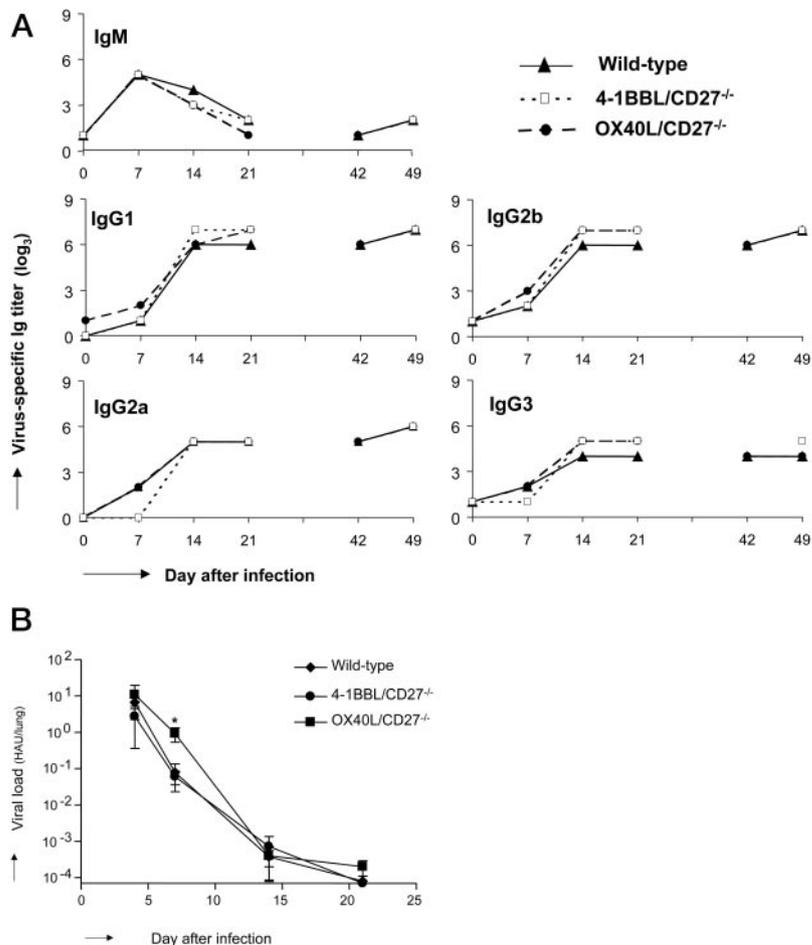


FIGURE 2. Ig responses and viral load in absence of CD27, 4-1BB, and/or OX40 signals. *A*, At the indicated days after infection, serum titers of the indicated virus-specific Ig isotypes in wild-type, 4-1BBL/CD27^{-/-}, and OX40L/CD27^{-/-} mice were determined by ELISA. Primary challenge was done at day 0, and secondary challenge at day 42. Each data point represents a measurement on pooled sera from five mice. Titers are expressed as outlined in *Materials and Methods*. *B*, Viral load in lungs of the indicated mice at days 4, 7, 14, and 21 after infection was determined by PCR. It is presented as means + SE in hemagglutinating units per lung. The limit of detection is at 6.95×10^{-5} hemagglutinating units/lung. Data are derived from three to four mice per group.

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

To compare the capacity of mice deficient for CD27, 4-1BBL, or OX40L to mount a memory CD8⁺ T cell response, mice were reinfected with the same virus 6 wk after the first challenge. The occurrence of H-2D^b/NP₃₆₆₋₃₇₄⁺ CD8⁺ T cells was determined 3, 5, 7, and 11 days after secondary infection. Characteristically, H-2D^b/NP₃₆₆₋₃₇₄⁺-specific T cells reached peak levels earlier in lungs of wild-type mice earlier than in the primary response (day 5 vs days 8–10) and were higher in number (Fig. 3). Strikingly, in this secondary response, all three receptor/ligand systems were required for accumulation of H-2D^b/NP₃₆₆₋₃₇₄⁺-specific T cells in the lung. In DLN and spleen, generation of H-2D^b/NP₃₆₆₋₃₇₄⁺ memory effector T cells was also reduced. CD27 deficiency had the most profound impact, while lack of 4-1BBL had some effect and lack of OX40L had almost no statistically significant consequences (Fig. 3).

Analysis of responses in 4-1BBL/CD27^{-/-} and OX40L/CD27^{-/-} double-deficient mice revealed complementarity between CD27 and 4-1BB or OX40. In lung, and particularly in DLN and spleen, accumulation of virus-specific CD8⁺ T cells was further reduced in double-deficient mice than in single-deficient mice (Fig. 3). These data demonstrate that the memory CD8⁺ T cell response to intranasal infection with influenza virus critically depends on the collective, partially nonredundant contributions that result from interaction among CD27, 4-1BB, OX40, and their ligands.

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

Accumulation of effector T cells in the memory response is determined by two key parameters: the number of memory T cells formed and the extent of their secondary expansion. To better understand the effect of CD27, 4-1BB, and OX40 on accumulation of virus-specific T cells in the memory response, we studied their impact on these parameters separately. First, we determined the effect of CD27, 4-1BBL, or OX40L deficiency on the generation of memory CD8⁺ T cells. In blood of wild-type mice, the percentage of T cells within the CD8⁺ population that stained with H-2D^b/NP₃₆₆₋₃₇₄ tetramers at 6 wk after primary infection was >7-fold higher than in naive mice, indicating the presence of memory (Fig. 4A). In mice deficient for CD27, 4-1BBL, or OX40L, CD8⁺ memory T cell levels were almost 2-fold reduced as compared with wild type, while in 4-1BBL/CD27^{-/-} and OX40L/CD27^{-/-} mice these numbers were a further 2-fold reduced, coming close to background levels in naive mice (Fig. 4A). In spleens of wild-type memory mice, H-2D^b/NP₃₆₆₋₃₇₄⁺ T cell numbers were increased ~6-fold as compared with numbers found in naive mice. In CD27^{-/-}, 4-1BBL^{-/-}, and OX40L^{-/-} mice, levels of H-2D^b/NP₃₆₆₋₃₇₄-specific memory T cells were reduced ~2-fold as compared with those in wild-type mice (Fig. 4B). Interestingly, in the lungs of previously infected mice, numbers of tetramer⁺ T cells were ~10-fold higher than in naive mice. These most likely represent tissue memory cells, because their adoptive transfer gave rise to a memory response (results not shown). As in the spleen, all

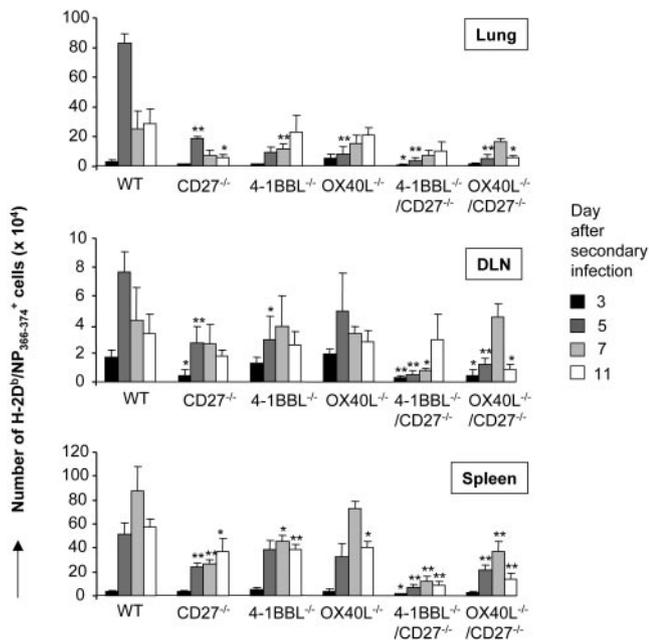


FIGURE 3. Memory CD8⁺ T cell responses in absence of CD27, 4-1BB, and/or OX40 signals. Six weeks after primary infection, mice of the indicated genotypes were reinfected with the same influenza virus strain. Cells were analyzed as outlined for Fig. 1. Bars represent mean values ($n = 4$); error bars indicate SEM. Two-tailed Student's t test indicated significant differences compared with wild-type values for $p \leq 0.05$ (*) and $p \leq 0.01$ (**). The experiments are representative of two.

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).

We conclude that after intranasal influenza virus infection, generation of the CD8⁺ central and tissue memory T cell pools relies on collective, nonredundant contributions of CD27, 4-1BB, and OX40. Although OX40L deficiency did not affect the accumulation of virus-specific 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. 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^{-/-}, 4-1BBL^{-/-}, or OX40L^{-/-} mice

To determine the contribution of CD27, 4-1BBL, and OX40L to expansion and accumulation of memory CD8⁺ T cells during the secondary response, we performed adoptive transfer experiments. A small sample of the T cells that were purified from spleens of primed wild-type, CD27^{-/-}, 4-1BBL^{-/-}, and OX40L^{-/-} was set aside and stained with tetramers. This allowed adjustment of the number of cells used for adoptive transfer, so that each recipient mouse received an equal amount of H-2D^b/NP₃₆₆₋₃₇₄-specific T cells (see Fig. 4 for numbers of memory cells in this experiment). In this way, we corrected for the effects of CD27, 4-1BBL, and OX40L deficiency on memory T cell formation, allowing us to monitor their effect on secondary expansion as such. Donor T cells were labeled with CFSE and injected into primed wild-type recipient mice. Their accumulation was analyzed at day 6 after secondary infection (Fig. 5A).

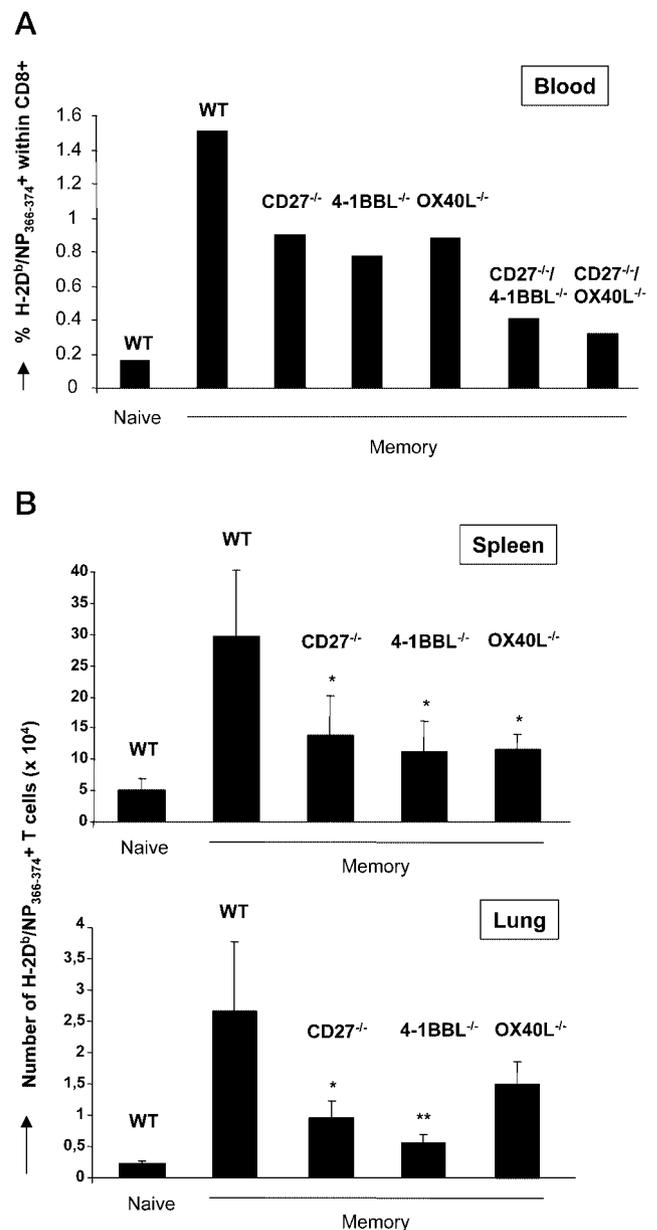
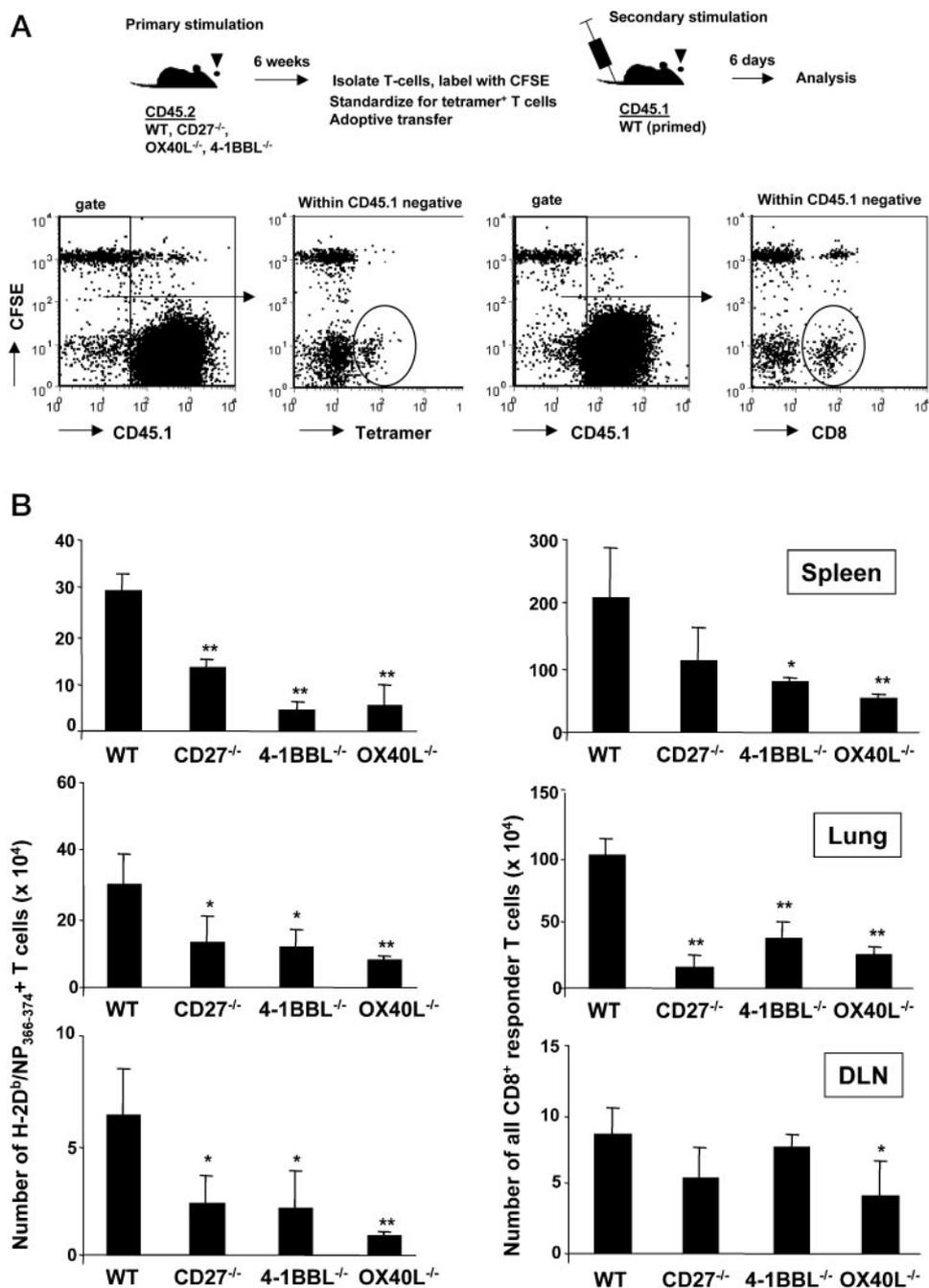


FIGURE 4. Formation of CD8⁺ T cell memory in absence of CD27, 4-1BB, and/or OX40 signals. **A**, Blood was collected from wild-type (WT) and recombinant mice at 6 wk after infection, as well as from naive WT mice ($n = 6$, pooled). Bars represent percentage of tetramer⁺ cells within the CD8⁺ population. **B**, Cells were harvested from spleens and lungs of WT, CD27^{-/-}, 4-1BBL^{-/-}, and OX40L^{-/-} mice at 6 wk after influenza infection ($n = 4$). Bars represent mean number of tetramer⁺ CD8⁺ T cells. Results are representative of two experiments.

In spleen, lung, and DLN, accumulation of H-2D^b/NP₃₆₆₋₃₇₄-specific T cells derived from primed CD27^{-/-}, 4-1BBL^{-/-}, or OX40L^{-/-} mice was significantly reduced as compared with 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^{-/-}, 4-1BBL^{-/-}, or OX40L^{-/-} 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 do not know to which extent the total CD8⁺ responder T cell pool represents bystander cells, but it very

FIGURE 5. Secondary expansion of CD8⁺ memory T cells from CD27^{-/-}, 4-1BBL^{-/-}, or OX40L^{-/-} mice in wild-type recipients. **A**, Experimental setup. Wild-type (WT), CD27^{-/-}, 4-1BBL^{-/-}, and OX40L^{-/-} mice of the CD45.2 allotype were infected with influenza virus. After 6 wk, T cells were purified from spleen, stained with H-2D^b/NP₃₆₆₋₃₇₄ tetramers and anti-CD8 mAb, and labeled with CFSE. T cell samples, standardized to contain equal numbers of tetramer⁺ T cells, were injected into primed CD45.1⁺ wild type. At day 6 after secondary infection, cells from spleens, lungs, and DLN were stained with anti-CD45.1 mAb, NP₃₆₆₋₃₇₄/H-2D^b tetramers, and anti-CD8 mAb, and analyzed by flow cytometry. Representative dot plots of WT spleen samples show gating on donor cells and the definition of responder cell by CFSE dilution in tetramer⁺ and total CD8⁺ populations. **B**, Absolute numbers of either tetramer⁺ or CD8⁺ responder T cells from the donor, calculated from the percentage of tetramer⁺/CD45.1⁻/CFSE⁻ or CD8⁺/CD45.1⁻/CFSE⁻ cells and the total number of cells per organ. Bars represent means (*n* = 4). The experiment shown is representative of two.



likely includes virus-specific T cells, because influenza virus contains other immunogenic MHC class I-restricted epitopes besides NP₃₆₆₋₃₇₄ (40). We conclude that memory CD8⁺ T cells specific for the H-2D^b/NP₃₆₆₋₃₇₄ complex, as well as memory CD8⁺ T cells with other virus specificities, are dependent on the collective contributions of CD27, 4-1BBL, and OX40L for their secondary expansion and establishment at the site of infection.

Memory CD8⁺ T cells do not require support by 4-1BBL and OX40L on non-T cells throughout the secondary response

In the experiment described above, memory T cells derived from 4-1BBL^{-/-} or OX40L^{-/-} mice normally expressed 4-1BB or OX40, while their ligands were present in the wild-type recipients. We considered therefore that absence of 4-1BBL or OX40L during the primary response might have compromised the intrinsic capacity of memory T cells from 4-1BBL^{-/-} and OX40L^{-/-} mice to

expand upon renewed challenge. Subsequent experiments were designed to address this possibility.

To test whether 4-1BBL or OX40L was required throughout the secondary response, we examined secondary expansion of wild-type memory cells in a ligand-deficient environment. T cells were purified from the spleen of primed wild-type mice, labeled with CFSE, and allowed to undergo secondary expansion in wild-type, 4-1BBL^{-/-}, or OX40L^{-/-} recipients. The response was analyzed as outlined for Fig. 5. It appeared that wild-type memory CD8⁺ T cells expanded and accumulated in spleen, lung, and DLN of 4-1BBL^{-/-} or OX40L^{-/-} recipients to a similar extent as in wild-type recipients (Fig. 6). Both H-2D^b/NP₃₆₆₋₃₇₄-specific and total CD8⁺ memory T cells were independent of 4-1BBL or OX40L for their accumulation. In this system, the only cells that may express 4-1BBL or OX40L are the transferred T cells themselves. We conclude, therefore, that memory CD8⁺ T cells do not require

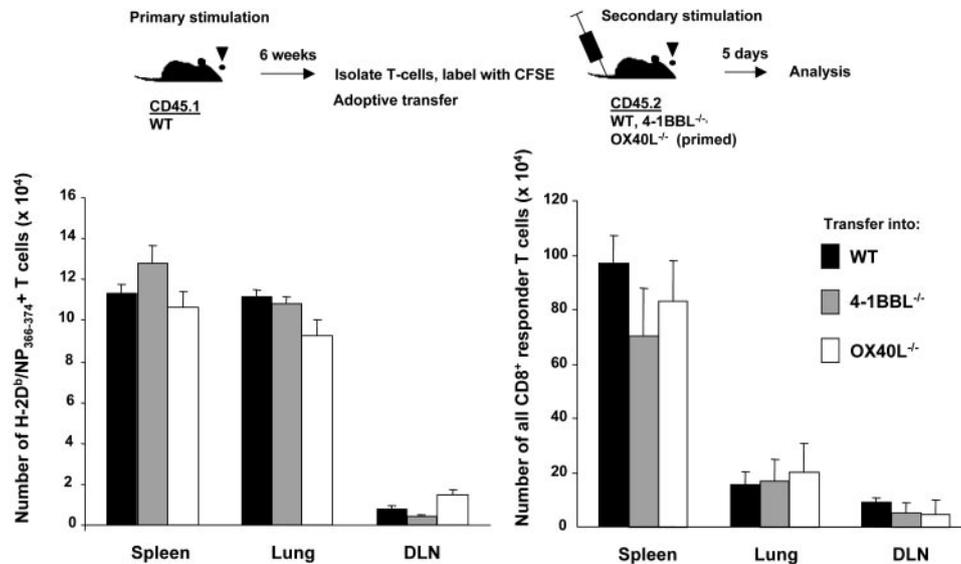


FIGURE 6. Secondary expansion of wild-type memory CD8⁺ T cells in 4-1BBL^{-/-} or OX40L^{-/-} recipients. Wild-type (WT) CD45.1⁺ mice were infected with influenza virus. Six weeks later, T cells were isolated from spleen, labeled with CFSE, and injected into primed CD45.2⁺ WT, 4-1BBL^{-/-}, or OX40L^{-/-} recipients. At day 5 after secondary infection, cells from spleens, lungs, and DLN were stained with anti-CD45.1 mAb, H-2D^b/NP₃₆₆₋₃₇₄ tetramers, and anti-CD8 mAb, and analyzed by flow cytometry. Absolute numbers of either tetramer⁺ or CD8⁺ responder T cells were calculated from the percentage of tetramer⁺/CD45.1⁺/CFSE⁻ or CD8⁺/CD45.1⁺/CFSE⁻ cells and the total number of cells per organ. Bars represent means ($n = 3$). The experiment shown is representative of two.

expression of 4-1BBL or OX40L on non-T cells throughout the secondary response for their expansion in spleen and DLN and for their accumulation in lung, the site of infection.

4-1BBL and OX40L 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 4-1BBL^{-/-} or OX40L^{-/-} 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 4-1BBL^{-/-} or OX40L^{-/-} recipients, monitored their conversion to CD8⁺ memory T cells, and subsequently let standardized numbers of memory cells respond to rechallenge in wild-type mice. In this experiment, we also tested the capacity of CD8⁺ T cells from the 4-1BBL^{-/-} and OX40L^{-/-} recipients to form memory and to respond to rechallenge in wild-type mice (Fig. 7A).

The capacity of wild-type CD8⁺ T cells to form memory in 4-1BBL^{-/-} or OX40L^{-/-} recipients (as monitored in blood) was reduced as compared with the wild-type situation (Fig. 7B). In fact, wild-type CD8⁺ T cells formed H-2D^b/NP₃₆₆₋₃₇₄-specific memory in 4-1BBL^{-/-} or OX40L^{-/-} recipients as inadequately as CD8⁺ T cells from these recipients themselves. Apparently, CD8⁺ T cells require stimulation of 4-1BB and OX40 by their ligands on non-T cells for memory formation.

As also shown in Fig. 5B, the secondary response of H-2D^b/NP₃₆₆₋₃₇₄-specific memory T cells, as well as total CD8⁺ T cells from 4-1BBL^{-/-} or OX40L^{-/-} first recipients in wild-type secondary recipients was impaired, as evident from their significantly decreased accumulation in spleen, DLN, and lung (Fig. 7C). Interestingly, wild-type CD8⁺ memory T cells primed in a 4-1BBL^{-/-} or OX40L^{-/-} environment were also impaired in secondary responsiveness (Fig. 7D). This was evident from the significantly reduced expansion of H-2D^b/NP₃₆₆₋₃₇₄-specific T cells in the spleen and their reduced accumulation in the lung. The de-

fect in the lung was also statistically significant for the total CD8⁺ T cell population (Fig. 7D). From this experiment, we derive the conclusion that triggering of 4-1BB or OX40 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 secondary challenge. The fact that secondary expansion of memory T cells from ligand-deficient mice was more severely affected than that of memory T cells from wild-type mice (Fig. 7, compare C and D) can be explained by a contribution of ligand on T cells to imprinting for secondary expansion and/or by undefined intrinsic differences between T cells of wild-type and ligand-deficient genotype.

Expression of receptors and ligands on T cells and APCs during antiviral responses

To understand where and when throughout the immune response to influenza virus CD27, 4-1BB, OX40, 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 Ab to the relevant receptor or ligand and to CD3 as T cell marker, CD19 as B cell marker, or CD11c as marker for myeloid cells, in particular DC. The expression of receptors and ligands was consistently most pronounced in the lung (Fig. 8), as compared with DLN and spleen (data not shown). With regard 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. 8A). On the days examined, 4-1BB was virtually undetectable on T cells, but quite prominent on CD11c⁺ cells in lung (Fig. 8A), DLN, and spleen (data not shown). OX40 was found on a minority of T cells, which were most abundant in the lung during the primary response (Fig. 8A). CD27 and OX40 were also present on CD11c⁺ cells in these organs, but to a lesser extent than 4-1BB (Fig. 8A, 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. 8A).

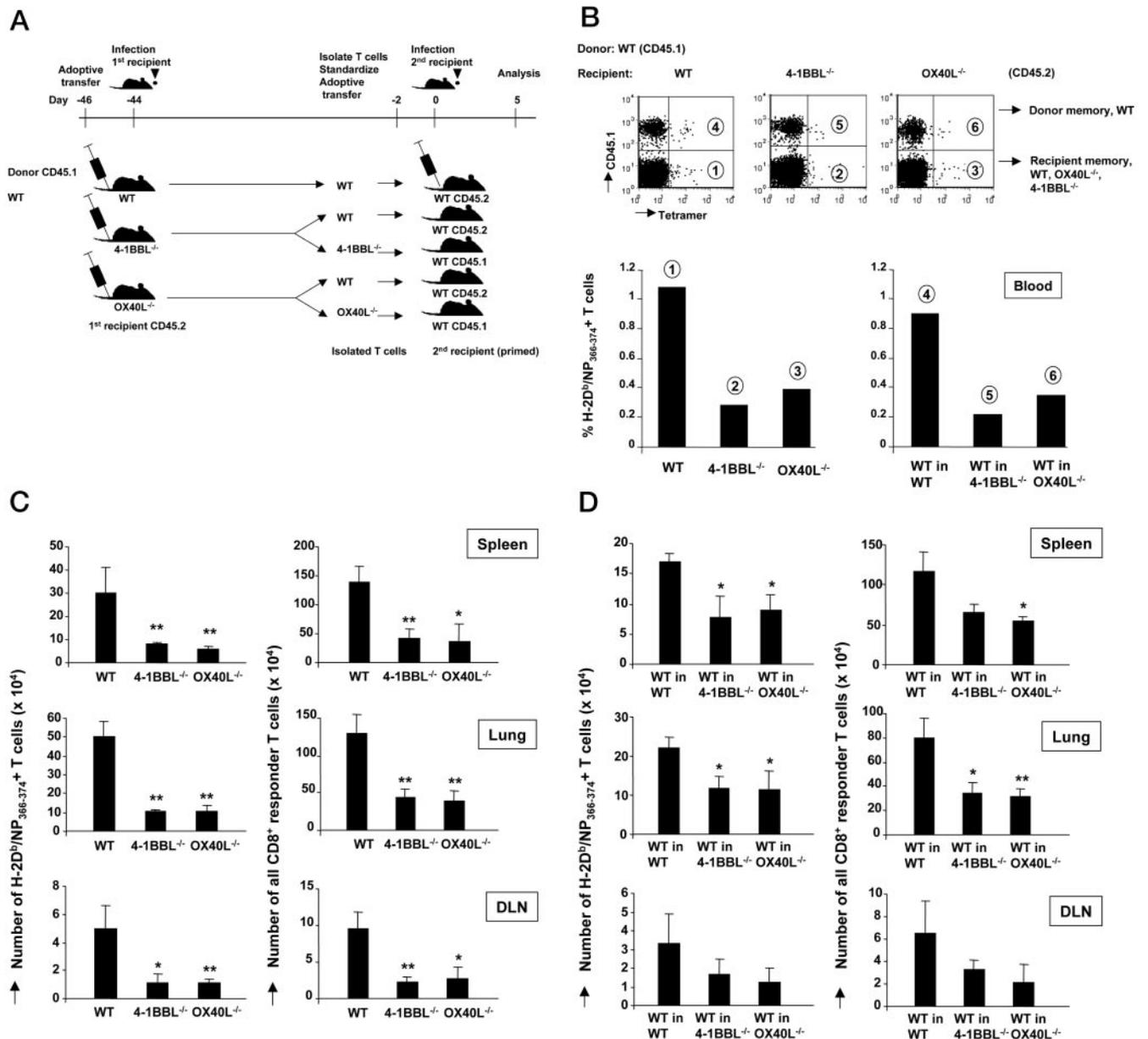


FIGURE 7. Wild-type T cells primed in absence of 4-1BBL or OX40L show defective secondary expansion. *A*, Experimental design. Purified splenic T cells from naive wild-type (WT) CD45.1⁺ mice were injected into naive CD45.2⁺ wild-type, 4-1BBL^{-/-}, or OX40L^{-/-} mice. At 6 wk after infection, T cells were purified from spleen and stained with anti-CD45.1 mAb; H-2D^b/NP₃₆₆₋₃₇₄ tetramers and anti-CD8 mAb to determine memory T cell formation. Next, populations of purified splenic T cells, standardized for numbers of tetramer⁺ T cells, were injected into primed WT secondary recipients. CD45.1⁺ T cells from the primary WT donors were injected into CD45.2⁺ recipients and CD45.2⁺ T cells from WT, 4-1BBL^{-/-}, or OX40L^{-/-} 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/NP₃₆₆₋₃₇₄ specificity in blood, as detected 6 wk after infection of the first recipients. Bars indicate percentage of tetramer⁺ cells ($n = 3$, pooled). *C* and *D*, At day 5 after secondary infection, cells from spleens, lungs, and DLN were stained with anti-CD45.1 mAb, tetramers, and anti-CD8 mAb, and analyzed by flow cytometry. Absolute numbers of either tetramer⁺ or CD8⁺ T cells were calculated, as outlined for Fig. 4. Bars represent means ($n = 3$). *C*, Shows the result for CD45.2⁺ T cells of the first recipient (WT, 4-1BBL^{-/-}, or OX40L^{-/-}); *D*, Shows the results for wild-type CD45.1⁺ T cells of the primary donor. The experiment shown is representative of two.

The ligands of the TNF family are notoriously difficult to detect because they are transiently expressed, contingent upon immune activation. We found CD70, 4-1BBL, and OX40L on T cells in DLN, spleen, and lung, but at low frequency. The ligands were most prominent on CD11c⁺ cells, while they were also found on B cells (Fig. 8*B*). From comparing expression of CD27, 4-1BB, OX40, and their ligands in the lung throughout primary and secondary responses, it appears that 4-1BB/4-1BBL and OX40/

OX40L are much less prominently expressed in the secondary response (Fig. 8, *A* and *B*).

The primary data in Fig. 8*C* highlight the transient and activation-specific nature of ligand expression, in this case on CD11c⁺ (DC) in the lung, the site where they were most abundant. Throughout the primary response, the ligands were acquired by CD11c⁺ cells in the lung and subsequently lost. Cell surface expression of CD70 was at all times very low, making the exact

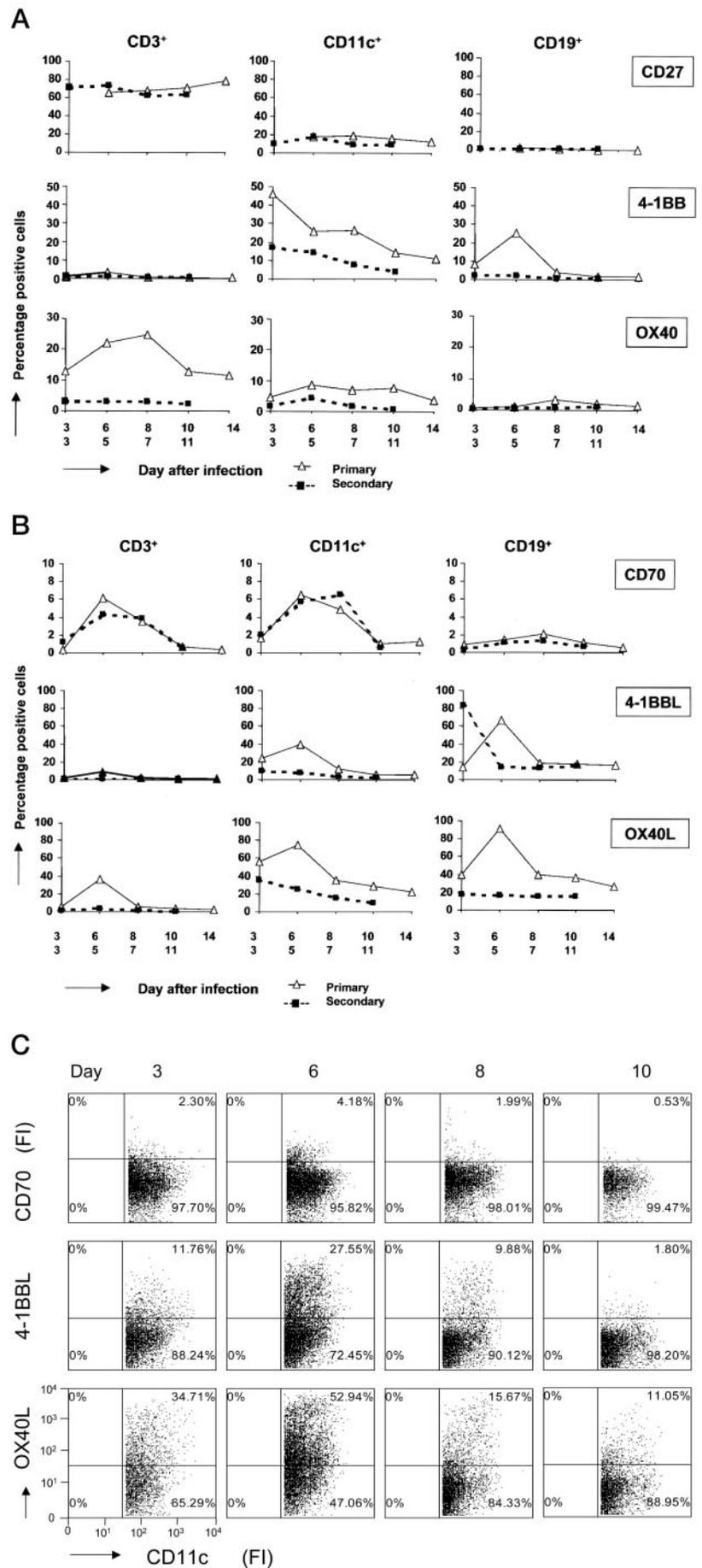


FIGURE 8. Expression of receptors and ligands in lung during primary and secondary infection. At the indicated time points after primary or secondary infection, cells extracted from lungs were stained with Abs for CD3, CD11c, or CD19; combined with Abs for CD27, 4-1BB, OX40, CD70, 4-1BBL, or OX40L; and analyzed by flow cytometry. *A*, Percentage of lung-infiltrating CD3⁺ cells (T cells), CD11c⁺ cells (enriched for DC), and CD19⁺ cells (B cells) expressing CD27, 4-1BB, or OX40. *B*, Percentage of these populations expressing CD70, 4-1BBL, or OX40L. *C*, Flow cytometry dot plots showing fluorescence intensity of CD70, 4-1BBL, and OX40L staining on gated lung-infiltrating CD11c⁺ cells.

percentage of positive cells difficult to determine. However, 4-1BBL and OX40L were expressed at high levels on large fractions of CD11c⁺ cells at day 6 after primary infection, a time when effector T cells start entering the lung (Fig. 8C). B cells in the lung also express significant levels of these ligands (Fig. 8B). These data suggest that CD11c⁺ cells (DC) and B cells at the effector site may play a role in regulating the size of the effector and memory T cell pools, as well as the functional properties of developing memory cells.

Discussion

In this study, we have determined that upon intranasal infection with influenza virus, CD27, 4-1BB, and OX40 collectively shape the same CD8⁺ T cell memory pool, both in terms of numbers of memory T cells formed and their capacity to accumulate upon secondary challenge. The contributions of CD27, 4-1BB, and OX40 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 4-1BB and OX40 endow memory CD8⁺ T cells during their formation with an improved capacity for secondary responsiveness.

As shown for OX40 receptor deficiency (29), 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 was the first *in vivo* evidence that OX40 can promote primary CD8⁺ T cell responses (33). A recent report monitoring anti-OVA responses of OX40-deficient CD8⁺ T cells corroborates this (34). In a model of *i.p.* injection with influenza virus (HKx31), virus-specific CD8⁺ T cell accumulation in the spleen during the effector phase was normal in mice lacking 4-1BBL, OX40L, or both (28, 35). 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 Bertram et al. (28), we found no contribution of 4-1BB/4-1BBL to the primary T cell response in the spleen. CD27 similarly did not affect the primary CD8⁺ T cell response in this organ. We suspect 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 naive 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 up-regulate different antiapoptotic molecules, which differ in their capacity to counteract certain death signals that operate in activated T cells. A third possibility is that CD27 and 4-1BB differ in their capacity to direct cellular responses unrelated to apoptosis.

With regard to the formation of memory T cells, it is well established that deliberate ligation of 4-1BB or OX40 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 for 4-1BB it was the other way around (22, 23). After *i.p.* challenge with influenza virus, 4-1BBL^{-/-} mice showed a reduction in virus-specific CD8⁺ T cells late in the primary response and upon rechallenge, while OX40L^{-/-} mice had no defects (28, 35). In this study, we show that upon intranasal delivery of influenza virus,

4-1BB, CD27, and OX40 collectively shape the same CD8⁺ T cell memory T cell pool. In the intranasal influenza virus model, absolute numbers of H-2D^b/NP₃₆₆₋₃₇₄ memory T cells are low, but tetramer staining is manifold over background levels found in naive 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, 4-1BBL, and OX40L to T cell memory in the circulation were comparable in magnitude. Given the similarity in viral clearing in wild-type and double-deficient mice, it is unlikely that viral loads have had a major differential impact on memory formation in the wild-type and recombinant strains.

Our data indicate that CD27, 4-1BBL, and OX40L contribute to central as well as tissue memory formation of CD8⁺ T cells. Interesting in this respect is that expression of CD70, 4-1BBL, and OX40L is most pronounced at the effector site. Unexpectedly, both CD11c⁺ cells and B cells in the lung carried these ligands. We suggest that communication between effector T cells and APCs (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 prosurvival contribution.

We had difficulty in detecting 4-1BB and OX40 on T cells *in vivo*, but *in vitro* studies have proven that these receptors directly transmit survival signals into T cells (6, 7). 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 affect T cell function indirectly.

An important aspect of our work is that we have separated effects of CD27, 4-1BB, and OX40 on memory formation from those on secondary expansion. This has been done using T cell populations, which were standardized for the amount of H-2D^b/NP₃₆₆₋₃₇₄-specific cells for adoptive transfer. This setup has allowed us to uncover that memory T cells do not need support by 4-1BBL or OX40L during secondary expansion, but require it for optimal programming during the primary response. One study that has used a similar approach documents normal secondary expansion of primed T cells from 4-1BBL^{-/-} mice in wild-type recipients (41). We believe that the difference may be due to the *i.p.* challenge with influenza virus that was used. As discussed above, this may involve a mode of priming (in the spleen) that bypasses the need for survival support by 4-1BB and its relatives. Because CD70-deficient mice are not available, we could not use a similar setup to test whether CD27 can imprint into CD8⁺ T cells the capacity for secondary expansion. However, a recent study using recombinant soluble CD70 for costimulation during primary challenge with OVA suggests that this might be the case (42).

An important subject of recent studies is the question as to whether T cell responses proceed according to a pre-established program after initial Ag encounter. TCR stimulation triggers a developmental program in naive CD8⁺ T cells, allowing them, in the

subsequent absence of Ag, to divide at least seven times, to develop cytolytic effector functions, and to acquire memory characteristics (43, 44). However, our data argue that Ag is an important factor in controlling T cell survival and the extent of memory formation. We postulate that when Ag wanes, CD70, 4-1BBL, and OX40L disappear and with them the prosurvival effects of their receptors. In such a scenario, Ag 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 antiapoptotic molecules. In the memory phase, survival support comes from cytokines such as IL-15 (45).

Evidence has been presented recently that CD4⁺ T cells can program the capacity for secondary expansion into CD8⁺ T cells during priming (46–48). Whether the effect of 4-1BB and OX40 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 CD8⁺ memory formation and responsiveness are exclusively regulated by 4-1BBL or OX40L on CD4⁺ T cells, because we have proven that ligands on non-T cells are also important for this. We do not know when the programming for secondary expansion occurs, or what it entails at a molecular level. Because memory T cells are slowly cycling (45), it must be a capacity that can be transmitted to the daughter cells and therefore can truly be termed programming. Our collective data strongly support the idea that deliberate offering of CD70, 4-1BBL, and OX40L during priming may be a good strategy to achieve potent and long-lasting immunity.

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Disclosures

The authors have no financial conflict of interest.

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