Temporal Segregation of 4-1BB Versus CD28-Mediated Costimulation: 4-1BB Ligand Influences T Cell Numbers Late in the Primary Response and Regulates the Size of the T Cell Memory Response Following Influenza Infection

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Temporal Segregation of 4-1BB Versus CD28-Mediated Costimulation: 4-1BB Ligand Influences T Cell Numbers Late in the Primary Response and Regulates the Size of the T Cell Memory Response Following Influenza Infection

Edward M. Bertram, Peggy Lau, and Tania H. Watts

In this report, we demonstrate that CD28−/− mice are severely impaired in the initial expansion of Db/NP366-374-specific CD8 T cells in response to influenza virus infection, whereas 4-1BB ligand (4-1BBL)−/− mice show no defect in primary T cell expansion to influenza virus. In contrast, 4-1BB−/− mice show a decrease in Db/NP366-374-specific T cells late in the primary response. Upon secondary challenge with influenza virus, 4-1BB−/− mice show a decrease in the number of Db/NP366-374-specific T cells compared to wild-type mice such that the level of the CD8 T cell expansion during the in vivo secondary response is reduced to the level of a primary response, with concomitant reduction of CTL effector function. In contrast, Ab responses, as well as secondary CD4 T cell responses, to influenza are unaffected by 4-1BB deficiency. Thus, CD28 is critical for initial T cell expansion, whereas 4-1BB/4-1BBL signaling affects T cell numbers much later in the response and is essential for the survival and/or responsiveness of the memory CD8 T cell pool. The Journal of Immunology, 2002, 168: 3777–3785.

D uring the initial stages of T cell activation, the simultane- ous engagement of the TCR and CD28 leads to organi- zation of the T cell signaling complex at the T cell- APC interface (1), the production of IL-2, and survival of the T cell (2). Once a T cell has reached the threshold for activation, it initiates a program of autonomous cell division without a require- ment for further antigenic stimulation (3–6). Subsequent to the initial productive interaction of a T cell with an Ag-bearing APC, a number of additional receptor-ligand pairs can be up-regulated on the T cell and APC surface, peaking at ~48–72 h after in vitro T cell activation (reviewed in Ref. 7). Several members of the TNFR family, including 4-1BB(CD137), OX40 (CD134), and CD27 have been shown to play a role in T cell activation subsequent to initial activation events (8–10). Given the recent evidence that once activated, T cells continue their activation program through several divisions in the absence of a further antigenic signal, the question arises as to when and how these activation-in- duced costimulatory receptors come into play.

4-1BB is a costimulatory member of the TNFR family ex- pressed on activated T cells (8). Upon engagement with 4-1BB ligand (4-1BBL)3 or aggregation with anti-4-1BB, 4-1BB can pro- vide a CD28-independent costimulatory signal leading to CD4 and CD8 T cell expansion, cytokine production, development of CTL effector function, and prevention of activation-induced death (11– 18). In vitro, resting CD28−/− T cells can up-regulate 4-1BB quickly enough to respond to 4-1BBL within 24 h of anti-CD3 treatment (15). In mixed lymphocyte reactions, 4-1BBL was found to increase T cell numbers, particularly at days 5–7 of stimulation, consistent with a role for 4-1BBL in T cell survival (18). 4-1BBL (19) is expressed on activated APC only after 2–3 days of activa- tion and is likely the limiting factor in 4-1BB-mediated costimul- atory responses (11). Thus, although 4-1BB can provide a CD28-independent costimulatory signal for resting T cells in vitro, in vivo it is likely to act later in the response. Initial analysis of 4-1BB−/− mice showed that 4-1BB plays a role in augmenting suboptimal anti-viral CTL responses, skin allograft rejection as well as MHC I- and MHC II-restricted graft-vs-host disease (16, 20–22). For influenza-specific responses, 4-1BBL−/− mice showed a partial defect in the in vitro secondary CTL response to influenza virus. In the case of lymphocytic choriomeningitis virus (LCMV), there was no defect in viral clearance or in the primary T cell response in 4-1BB−/− mice in one study (16). However, in another study, it was found that there was a 2-fold decrease in Ag-specific T cell numbers, measured at day 8 after infection (21). Under conditions of suboptimal priming using a lipitated LCMV peptide, 4-1BBL−/−, but not wild-type, mice succumbed to challenge with a more virulent strain of LCMV (21). In vitro studies using LCMV-specific P14 transgenic T cells showed 4-1BBL-de- pendence of the LCMV-specific response only when a weak ago- nist peptide was used (16). Thus, there is accumulating evidence that 4-1BBL can influence both primary and secondary virus-spe- cific CTL responses.

To pinpoint the time at which 4-1BB plays a role during the response to influenza infection, in this report we have followed specific T cell expansion in wild-type, CD28−/−, and 4-1BB−/− mice following primary and secondary infection. The results pre- sented show that CD28 is critical for primary expansion of influenza-specific CD8 T cells, whereas 4-1BB has an impact only late in the primary response, thereby influencing the size of the memory pool and the subsequent secondary CD8 T cell response.
Materials Methods

Mice
C57BL/6 mice were bred in our facility from breeder pairs obtained from Charles River Breeding Laboratories (St.-Constant, Quebec, Canada). 4-1BBL−/− mice were also bred in our facility after backcrossing with C57BL/6 breeders (n = 46 crossings) and being screened as previously described (16). Following the final backcross, 4-1BBL−/− mice, as well as wild-type siblings from the same F₂ cross, were used. No differences were observed between C57BL/6 mice from Charles River Breeding Laboratories and 4-1BBL−/− litter mates bred in our facility, with respect to the number of tetramer-positive T cells observed 7 days after influenza infection (data not shown). CD28−/− mice backcrossed onto the C57BL/6 background were provided by Dr. T. Mak (Amgen Institute, Toronto, Canada) and were bred in our facility.

Influenza virus infection

Most studies of influenza virus infection of mice have used intranasal infection of naive mice or mice primed i.p. with virus. Low numbers of D0/1NP366-374-specific CD8 T cells are detected in the spleen and bronchoalveolar lavage of mice during primary intranasal HXK31 infections, with the peak of the response at day 8 (23). Pilot experiments using i.p. infection of C57BL/6 mice with influenza A HXK31 of wild-type mice resulted in highly reproducible numbers of D0/1NP366-374-specific CD8 T cells responding in the spleen, with the response peaking at day 7 after primary infection (average, 7% of CD8 T cells). This allowed us to study subtle differences in the 4-1BBL−/− vs wild-type mice during the primary response to influenza infection. Therefore, we continued with i.p. infections throughout the study. Seven- to 10-wk-old mice were infected with 200 hemagglutinin units (HAU) of influenza A HXK31 (H3N2) produced as described (24). This virus does not replicate extensively when given to mice i.p. At 3 wk postinfection, some mice were challenged with the same strain (HXK31) or with the serologically distinct A/PR/34 (PR8, H1N1) which shares the NP gene with HXK31. Mice were sacrificed at the indicated time points and their spleens were harvested for single cell suspensions. Although the CD28−/− mice showed a greatly reduced NP366-374-specific CD8 T cell response to influenza virus, none of the mice showed any obvious ill effects of virus infection.

Flow cytometry

Tetramer staining. Spleen cell suspensions were prepared in PBS/2% FCS/0.01% sodium azide on ice. Cells were surface-stained with PE-conjugated anti-CD8, FITC-conjugated anti-mouse CD44 (eBioscience, San Diego, CA), and APC-labeled tetramers consisting of murine class I MHC molecule H-2Db, β2-microglobulin, and influenza nucleoprotein peptide, NP366-374 (National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility, Atlanta, GA). For each experiment, appropriate isotype control mAbs were used.

Intracellular IFN-γ staining. Spleen cell suspensions were restimulated in culture medium (RPMI/10% FCS with antibiotics and 2-ME) for 6 h at 37°C with 1 μM NP366-374 peptide and GolgiStop (BD PharMingen, San Diego, CA). Cells were harvested, resuspended in PBS/2% FCS/azide, and surface-stained with PE anti-CD8 and FITC anti-CD62L as described above. Following surface-staining, cells were fixed in Cytofix/Cytoperm solution (BD PharMingen) and then stained with A4-conjugated anti-mouse IFN-γ or FITC-diluted in 1× perm/wash solution (BD PharMingen). Samples were analyzed using a FACSCaliber and CellQuest software (BD Biosciences, Mountain View, CA).

Cytotoxicity assay

Wild-type siblings, 4-BBL−/−, and CD28−/− mice were infected with 200 HAU influenza A HXK31 as described above. Splenocytes were harvested after 3 wk and restimulated in vitro by the addition of 100 nm of the H-2Dd-restricted peptide NP366-374 to 1-ml cultures containing 5 × 10⁶ spleen cells. On day 5, cells were resuspended to 0.5 ml and serial 3-fold dilutions of effectors were performed (referred to as dilution of 3) for 6 h at 37°C with 1 μM NP366-374 peptide and 1000 units/ml IFN-γ. Cell viability was assessed by trypan blue dye exclusion. The percentage of specific lysis was calculated from the equation: (experimental ⁵¹Cr release − spontaneous ⁵¹Cr release) ÷ (maximum ⁵¹Cr release − spontaneous ⁵¹Cr release) × 100% = % specific lysis.

Detection of influenza-specific Abs

Neutralizing Abs were measured by serial 2-fold dilutions of serum with 500 HAU/ml influenza HXK31 in 96-well round-bottom plates at 37°C for 45 min and compared with normal mouse serum. Samples were then added to 0.5% washed chicken red blood cells in 96-well round bottom plates. The titer of neutralizing Ab was the last dilution which blocked hemagglutination. Results are presented as the number of 2-fold dilutions above normal mouse serum which block hemagglutination.

Influenza A-specific Abs were determined for IgM, IgG1, and IgG2a isotypes. Each well of the 96-well plates were coated with 100 μl of 500 HAU/ml influenza A HXK31 in PBS for 1 h at 37°C followed by incubation at 4°C overnight. Plates were blocked with 5% skim milk in PBS/0.1% Tween 20 for 2 h at 37°C. Five-fold serial dilutions of serum in PBS/0.1% Tween 20 containing 5% skim milk were added to wells overnight at 4°C. After washing in PBS/0.1% Tween 20, HRP-conjugated anti-isotype Abs (Caltag Laboratories, Burlingame, CA) were added for 2 h at 37°C. Following washing, H₂O₂ and ABTS (Sigma-Aldrich, St. Louis, MO) were added in citrate phosphate buffer (pH 5.0) and color development was measured after 20 min at OD₄₅₀.

Cytokine assays

Spleen cells (5 × 10⁶ cells) were incubated with 250 HAU/ml of heat-killed (56°C, 30 min) influenza A HXK31 for 48 h. Supernatants were taken and the levels of IL-2 and IFN-γ were measured. IL-2 was detected using the indicator cell line CTLL ligand as described (12). Serial dilutions of culture supernatant were prepared in duplicate and incubated with 1 × 10⁴ indicator cells in 100 μl in 96-well plates for 24 h. During the final 6 h, the cells were labeled with [¹¹²]Itymiodine (Amer sham, Baie d’Urfe, Quebec, Canada). Cultures were harvested and analyzed on the Topcount 96-well liquid scintillation counter (Canberra Packard). ELISA was performed on diluted supernatants from cultures using pairs of anti-murine IFN-γ mAbs purchased from BD PharMingen according to the manufacturer’s instructions.

Results

Analysis of the primary T cell response to influenza virus in the absence of CD28 or 4-1BBL

The immune response of mice infected with influenza HXK31 has been well studied (23, 25). The most important immune effectors during the primary response are the virus-specific CD8 T cells, with the NP366-374 epitope being immunodominant in C57BL/6 mice (26). To assess the role of the 4-1BB/4-1BBL signaling pathways during the immune response to influenza, wild-type siblings, 4-1BBL−/−, or CD28−/− mice were infected i.p. with 200 HAU of influenza virus A strain HXK31. Mice were sacrificed at different times following infection and spleen cells were analyzed for the number of Ag-specific cells using fluorescently-labeled D9/1NP366-374 tetramers. Initial experiments with heterozygous 4-1BBL−/+ mice showed no difference from their wild-type littersmates, so subsequent experiments were done only with +/- or −/− mice. The primary expansion of D9/1NP366-374-specific CD8 T cells was readily detected in wild-type mice following infection with influenza HXK31, with a peak response at 7 days postinfection (Fig. 1A). The responding CD8 T cells were CD62Llow, consistent with cells being of the activated/memory phenotype (Fig. 1C). 4-1BBL−/− mice showed a similar rate and magnitude of expansion of D9/1NP366-374-specific CD8 T cells during the primary response to influenza virus, indicating that there is no defect in initial T cell expansion in the absence of 4-1BBL. The 4-1BBL−/− interaction. The results in Fig. 1 are expressed as the percentage of CD8 T cells staining with tetramer. Conversion of these numbers to total tetramer-positive T cells per spleen, based on cell recoveries from spleen, gave identical results. There was no overall change in CD8 T cell recoveries from infected vs wild-type mice, likely due to the fact that influenza does not replicate extensively following i.p. infection of mice. Seven percent of CD8 T cells at day 7 corresponded to 10⁵ cells in the spleen for both wild-type and 4-1BBL−/− mice. In contrast, CD28−/− mice responded poorly to influenza virus, showing a substantial decrease...
Results are reported as the percentage of CD8 T cells staining with D\textsuperscript{b}/NP366-374 tetramer at 5 or 7 days. in influenza A virus strain HKx31 and sacrificed at 5 or 7 days. A, Cells were isolated from the spleen, counted, and stained with mAb to CD8, CD62L (CD62L), and rmH-2D\textsuperscript{b} tetramers, and analyzed by flow cytometry. Results are reported as the percentage of CD8 T cells staining with D\textsuperscript{b}/NP366-374 tetramer. B, Cells were restimulated with NP366-374 peptide for 6 h in the presence of GolgiStop and then stained with mAb to CD8 and CD62L before intracellular staining for IFN-\gamma. In A and B, each data point represents a single mouse with the average marked with a line; data were accumulated over three separate experiments. C, Example of flow cytometry plot at day 7 following influenza A infection. Panels shown are gated on live CD8\textsuperscript{+} T cells. The numbers in the upper right quadrants indicate the percentage of CD8 T cells staining with tetramer.

To monitor the development of effector function in the T cells following influenza virus infection of mice, splenocytes from the same infection described in Fig. 1A were also analyzed by intracellular staining for the production of IFN-\gamma, after a 6 h restimulation with NP366-374 peptide (Fig. 1B). Although the number of IFN-\gamma-producing CD8 T cells detected was consistently less than the number of tetramer-positive cells observed at each time point, the results were qualitatively similar to those obtained with D\textsuperscript{b}/NP366-374 tetramer staining. Thus, 4-1BBL\textsuperscript{−/−} mice show no defect in the number of IFN-\gamma-producing NP366-374-specific CD8 T cells following primary influenza infection. As was seen using tetramer staining, CD28\textsuperscript{−/−} mice also showed a large decrease in the number of NP366-374-specific IFN-\gamma-secreting CD8 T cells following influenza infection.

By day 21 postinfection, the number of D\textsuperscript{b}/NP366-374-specific CD8 T cells declines to 1.5% of the CD8 T cell population which corresponded to 2.2 \times 10^5 cells/spleen in wild-type mice (Fig. 2A).

This population of virus-specific CD8 T cells is thought to represent the pool of “memory” T cells that have avoided the cell death that follows the initial wave of T cell expansion. Although the differences detected were small, we consistently observed one-third lower numbers of D\textsuperscript{b}/NP366-374-specific CD8 T cells in 4-1BBL\textsuperscript{−/−} mice which had declined to 1.0% of the CD8 T cell population or 1.4 \times 10^5 cells/spleen. 4-1BBL\textsuperscript{−/−} mice also had slightly lower numbers of IFN-\gamma-producing cells compared with wild-type mice (0.81 ± 0.18% vs 1.27 ± 0.22%), consistent with the difference seen in tetramer staining at that time point (Fig. 2B).

To further explore the difference in T cell numbers late in the primary response, we carried out additional experiments in which we waited up to 38 days before analyzing NP366-374-specific T cell numbers (Fig. 2, A and C). Comparing the results at day 38 with those at day 21, it can be seen that in wild-type mice, the T cell numbers remain higher than those seen in 4-1BBL\textsuperscript{−/−} mice (1.04 vs 0.59%). Although small, the differences between wild-type and 4-1BBL\textsuperscript{−/−} mice at days 21 and 38 were found to be statistically significant (p < 0.01). These data suggest that 4-1BBL influences the size of the memory T cell pool late in the
To monitor the effect of 4-IBB/4-1BBL vs CD28/B7-mediated co-stimulation on CD8 T cell responses following in vitro restimulation with NP366-374.

Role of 4-1BBL vs CD28 on CTL responses following in vitro restimulation with NP366-374

To correlate the number of D^b/NP366-374-specific T cells observed in vivo with the acquisition of CTL effector function, as measured in vitro, splenocytes from mice infected 21 days previously with influenza virus HKx31 were restimulated with NP366-374 for 5 days. At the end of restimulation, splenocytes were assayed for their ability to lyse NP366-374-labeled syngeneic target cells (Fig. 3A). At limiting E:T ratios, ~3-fold more CTL effectors were required from the 4-1BBL^-/- mice to give equivalent killing compared with effectors from wild-type litter mates. As previously observed (16), CD28^-/- mice were severely impaired in their recall CTL response to influenza virus and this correlated with the failure to expand CD8^+ T cells in the primary response, resulting in a limited in vitro secondary response. At the same time as the CTL assay, the number of D^b/NP366-374-specific tetramer-positive and IFN-γ-producing CD8^+ T cells were measured (Fig. 3, B and C). Restimulated cultures from influenza-infected 4-1BBL^-/- mice had approximately two-thirds the number of tetramer-positive or IFN-γ-producing CD8^+ T cells as observed in cultures from wild-type mice. Thus, although there had been a large expansion of D^b/NP366-374-specific cells in cultures from influenza-infected wild-type and 4-IBBL^-/- mice, the relative defect observed at day 21 following primary infection is maintained following in vitro expansion of splenocytes from the mice.

Secondary CD8 T cell responses

To monitor the effect of 4-IBB/4-1BBL vs CD28/B7-mediated co-stimulation during the in vivo secondary response to influenza virus, mice which had been infected with influenza A strain HKx31 21 days previously were given a second dose of influenza A T cell expansion was measured at days 5 and 7 postinfection using D^b/NP366-374 tetramer staining of splenocytes directly ex vivo (Fig. 4A) or by intracellular staining for IFN-γ following restimulation with peptide (Fig. 4B).

To counter the effect of the neutralizing Ab response to HKx31 on the secondary CD8 T cell response, the challenge experiment used a different serotype of influenza A, the influenza A PR8 virus. The NP366-374 epitope is present in both HKx31 (H3N2) and PR8 (H1N1) influenza strains, which share the same NP gene. However, HKx31 and PR8 have different surface hemagglutinin and neuraminidase glycoproteins, so there is negligible cross-reactivity at the level of neutralizing Ab. Challenge of influenza HKx31-immune mice with PR8 virus leads to a secondary CD8^+ T cell response in the context of a primary Ab response (23, 25). The influenza-specific CD8 T cell response following PR8 challenge was monitored by following the number of D^b/NP366-374-specific CD8 T cells as well as the number of IFN-γ-producing CD8 T cells at days 5 and 7 postinfection (Fig. 4B). The expansion of D^b/NP366-374-specific CD8 T cells upon secondary infection was more rapid during the secondary response as indicated by the
greater number of tetramer-binding cells at the 5-day time point. In contrast to the results observed in the primary response, during the secondary response to influenza, 4-1BBL−/− mice showed a substantial decrease in the number of D9/NP366-374-specific T cells at days 5 and 7 postsecondary infection. Wild-type mice contained an average of 16% of CD8 T cells which corresponded to 2.3 × 10⁶ cells/spleen at day 5 following primary PR8 infection, whereas 4-1BBL−/− mice only had 7% of CD8⁺ T cells which corresponded to 10⁶ cells per spleen (this difference was statistically significant, p < 0.001). The primary response to influenza PR8 is similar to the primary response to influenza x31, resulting in 7% of CD8 T cells staining with the D9/NP366-374 tetramer (data not shown). Thus, the secondary response to influenza in 4-1BBL−/− mice is reduced to the level of a primary response. The defect in cell numbers in 4-1BBL−/− mice was also observed by monitoring intracellular IFN-γ staining, although as seen in the primary response, the numbers were lower overall.

Fig. 5 summarizes the time course of the immune response to influenza virus in wild-type siblings, 4-1BBL−/− or CD28−/− mice during the in vivo immune response to influenza virus, using tetramer staining measured at days 5, 7, 10, 14, 21, and 38 after primary infection and days 5 and 7 after secondary infection. The results show a delay in the effect of 4-1BB/4-1BBL-mediated costimulation, with CD28 influencing cell numbers early, and 4-1BBL influencing cell numbers only late in the primary response. As expected, mice given a second dose of the same influenza strain Hkx31 showed a reduced number of tetramer and IFN-γ-staining cells compared with PR8, presumably due to the neutralizing Ab reducing the infectious viral load (Fig. 5). Again, 4-1BBL−/− mice showed reduced responses compared with wild-type mice. This condition under both a high and low viral load, 4-1BBL−/− mice show a reduced CD8 T cell memory response to influenza virus. CD28−/− mice responded poorly to secondary infection with either PR8 or Hkx31 consistent with their greatly diminished primary response to influenza.

CD4 T cell responses to influenza infection of WT, CD28−/−, or 4-1BBL−/− mice

The effects of 4-1BBL could be due to a direct effect on CD8 T cells or perhaps indirect through CD4 T cells or by changing the levels of neutralizing Ab which would alter the availability of ineffective virus and the CTL response. To address this issue with CD4 T cells, we analyzed both late primary (day 38) and secondary CD4 T cell responses to influenza by restimulating T cells from immunized mice with heat-inactivated virus and measuring IL-2 and IFN-γ production (Fig. 6). In contrast to the results observed late in the primary and in the secondary CD8 T cell responses to influenza, there was no defect in CD4 T cell responses at either time point. In the same experiment, CD28−/− mice showed a complete lack of responsiveness in terms of IL-2 and IFN-γ production.

Ab responses to influenza in wild-type, CD28−/−, or 4-1BBL−/− mice.

Previous studies on 4-1BBL−/− mice showed no defect in the neutralizing Ab response to vesicular stomatitis virus (16) or in levels of LCMV-specific Abs in serum (20). However, treatment with agonistic anti-4-1BB Abs has been shown to interfere with humoral immunity, suggesting a role for 4-1BB in regulation of Ab responses (27). To address this issue in the context of influenza virus infection, we measured the neutralizing Ab response as well as the levels of influenza-specific Abs in wild-type vs CD28−/− or 4-1BBL−/− mice (Fig. 7). Both wild-type and 4-1BBL−/− mice produced similar amounts of neutralizing Ab to influenza A Hkx31 during the infection (Fig. 7A). The initial response was predominantly IgM at day 7 with class switching to IgG1 and
the serum samples were measured by ELISA as described in The data presented are the average mice at each time point. NMS, normal mouse serum.

2-fold dilutions above normal mouse serum that still blocks the hemagglutinating chicken red blood cells. Data is presented as the number of influenza A HKx31 from samples were measured by their ability to block in

FIGURE 7. Ab responses to influenza A are indistinguishable between WT and 4-1BBL−/− mice. Wild-type, 4-1BBL−/−, and CD28−/− mice were infected i.p. with 200 HAU influenza A virus strain HKx31 and sera was taken at days 7 and 38 and also at day 7 following secondary infection with influenza A HKx31. A. The levels of neutralizing Ab in the serum samples were measured by their ability to block influenza A HKx31 from agglutinating chicken red blood cells. Data is presented as the number of 2-fold dilutions above normal mouse serum that still blocks the hemagglutination. B. The isotypes of the Ab specific for influenza A HKx31 from the serum samples were measured by ELISA as described in Materials and Methods. The data presented are the average ± SEM of four individual mice at each time point. NMS, normal mouse serum.

IgG2a late in the primary and in the secondary response to influenza Hkx31 (Fig. 7B). CD28−/− mice initially produced neutralizing Ab at levels similar to wild-type mice at day 7 (Fig. 7A) and as expected this was predominantly IgM (Fig. 7B). However, late in the primary response, less neutralizing Ab was produced in CD28−/− mice compared with wild-type mice, correlating with low levels of influenza-specific IgG1 and IgG2a (Fig. 7, A and B). Thus, there is no defect in influenza-specific Ab responses in 4-1BBL−/− mice under conditions where a defect is observed in the CD28−/− mice. Taken together, the data suggest that the defect in the 4-1BBL−/− mice is restricted to CD8 T cells.

Discussion
The results presented in this report show that CD28 is required for the initial expansion of D9/NP366-374-specific CD8 T cells during the primary immune response to influenza virus. In contrast, 4-1BBL plays no detectable role in this initial T cell expansion but impacts on the memory response to influenza virus, such that upon secondary challenge, the expansion of CD8 tetramer-positive T cells is reduced to the level of the primary response. This effect could be due to an impact of 4-1BB only on secondary T cell expansion or could also be due to loss of memory T cells or induction of nonresponsiveness in the memory cells that survive following initial T cell expansion or a combination of these effects. We think an effect only on the secondary response is unlikely because 4-1BB is clearly up-regulated during primary as well as secondary activation of transgenic T cells (17, 18). Furthermore, the observation that the level of the secondary response in the absence of 4-1BBL is reduced to that of the primary T cell response is consistent with the idea that there has been a loss of memory T cell response. In support of this hypothesis is the finding that Ag-specific T cell numbers late in the primary response are reduced, arguing that the failure to see an enhanced in vivo secondary response to influenza virus in 4-1BBL−/− mice is due at least in part to a loss of memory T cells. Similarly, there is a correlation between numbers of tetramer-positive cells late in the primary response and the number of tetramer-positive cells obtained after restimulation in vitro (Fig. 3).

Previous work had demonstrated that 4-1BBL−/− mice are impaired in secondary responses to influenza virus, as measured by assaying cytotoxic T cell function upon restimulation in vitro. However, it was not clear at what point in the response the defect in the 4-1BBL−/− mice emerged (16). In the present study, by following Ag-specific T cell numbers using MHC I tetramers or IFN-γ staining, we found that 4-1BBL−/− mice have no defect in initial T cell expansion in response to influenza virus, rather the defect is observed much later in the response.

In vitro, 4-1BB is up-regulated on T cells with peak expression at 48–72 h after peptide-specific T cell activation and the expression is transient (17, 18). In vivo, immunization of mice with the superantigen staphylococcal enterotoxin A leads to peak expression of 4-1BB on CD8 T cells at 12 h with declining expression by 24 h (28). We have been unable to detect 4-1BB expression on the tetramer-positive T cells during primary or secondary infection with influenza likely due to the low level and transient nature of its expression (data not shown). However, the available evidence suggests that 4-1BB is expressed only early and transiently during the immune response in vivo. These data invoke a model in which signals delivered through 4-1BB early in the response impact on cell numbers much later in the primary response. Such an effect would be observed if 4-1BB impacted on signals involved in long-term T cell survival or differentiation into a “memory” cell. This model is conceptually similar to the work of Huang et al. (29) in which short-term IL-4 exposure during TCR stimulation results in induction of long-term memory of CD8+ T cells. Similarly, as mentioned in the introduction, several groups have shown that a short-term exposure to Ag initiates a program of events, resulting in several cycles of cell division (3–6). These types of experiments imply that signals given early during T cell activation can impact on the long-term fate of T cells. Based on the kinetic data shown here, we suggest that 4-1BB engagement early in T cell activation may impact on the efficiency with which the T cells enter or survive in the memory pool. The cell numbers, at days 21 and 38 of the primary response, show small but statistically significant differences (p < 0.01) between wild-type and 4-1BBL−/− mice and are consistent with a loss of memory T cells late in the primary response.

The number of Ag-specific T cells present at a given time point after viral infection reflects the net effects of expansion and death. Thus, 4-1BB could influence the size of the memory T cell pool by influencing the amount of cell division, the number of cells which undergo activation induced cell death following initial expansion, or could impact on the gradual loss of cells from the memory T cell.
pool over time. Hurtado et al. (13) have shown that anti-4-1BB treatment in vitro can prevent activation-induced cell death of repetitively activated T cells. However, in the present study examination of the loss of tetramer-positive cells immediately after the peak of the response shows no evidence for a major impact of 4-1BB at days 10–14 after infection, a time when the activated effector cells are being rapidly lost from the T cell pool. Furthermore, we did not observe differences in annexin V staining on tetramer-positive cells from influenza-infected wild-type or 4-1BB$^{-/-}$ mice (data not shown). Differences in T cell numbers between wild-type and 4-1BB$^{-/-}$ mice were statistically significant at days 21 and 38 after influenza infection. This might reflect a gradual loss of the memory T cells that have survived the initial activation-induced cell death that follows the expansion of the effector T cell pool.

The loss of tetramer$^+$ CD62L$^{low}$ memory CD8 T cells late in the primary response could be due to a direct effect of 4-1BBL on long-term survival of the CD8$^+$ influenza-specific T cells or it could be due to an indirect effect of CD4 help on the maintenance of the CD8 T cell response. CD4 T cells have been shown to respond to 4-1BBL in vitro (12, 13, 17, 18), and an effect of 4-1BB has been implied during MHC II-restricted graft-vs-host disease in vivo (22). However, we did not detect any decrease in secondary CD4 T cell responses to influenza in these mice (Fig. 6), arguing against a major role for 4-1BBL in the CD4 T cell response to influenza virus. Similarly, Tan et al. (20) observed little or no difference in the CD4 T cell response to LCMV in 4-1BB$^{-/-}$ vs wild-type mice. Thus, although 4-1BBL clearly can participate in activation of CD4 T cells in some experimental models, in the wild-type mice. Thus, although 4-1BBL clearly can participate in response to LCMV in 4-1BB$^{-/-}$ mice. Furthermore, there seemed to be a general defect in expansion of the loss of tetramer-positive cells immediately after the peak of the response shows no evidence for a major impact of 4-1BB at days 10–14 after infection, a time when the activated effector cells are being rapidly lost from the T cell pool. Furthermore, we did not observe differences in annexin V staining on tetramer-positive cells from influenza-infected wild-type or 4-1BB$^{-/-}$ mice (data not shown). Differences in T cell numbers between wild-type and 4-1BB$^{-/-}$ mice were statistically significant at days 21 and 38 after influenza infection. This might reflect a gradual loss of the memory T cells that have survived the initial activation-induced cell death that follows the expansion of the effector T cell pool.

In contrast to the effects of 4-1BBL on influenza virus, the response to LCMV in 4-1BB$^{-/-}$ mice was initially thought to be relatively unimpaired. DeBenedette et al. (16) showed no defect in the primary or secondary CTL response in 4-1BB$^{-/-}$ or 4-1BBL$^{-/-}$ CD8$^+$ T cells at day 7 with no detect in viral clearance. Tan et al. (20) also found that 4-1BB$^{-/-}$ mice cleared virus normally. However, when they analyzed the 4-1BBL$^{-/-}$ mice during the immune response to LCMV using tetramers specific for several viral epitopes, they found a small change in the percentage of CD8$^+$ T cells responding to LCMV at day 8 following infection (for example, 17% of CD8$^+$ T cells stained with GP33–41/Db tetramers at day 8 following infection, compared with 12% in 4-1BBL$^{-/-}$ mice). Furthermore, there seemed to be a general defect in expansion of the CD4$^{high}$ subset of CD8$^+$ T cells in the LCMV-infected mice, such that there were 2-fold more CD4$^{high}$ CD8$^+$ T cells in wild-type vs 4-1BBL$^{-/-}$ mice at day 8 following LCMV infection. In a subsequent study in which a lipidated peptide was used to immunize mice, Tan et al. (21) found a severe defect in the memory response to LCMV in 4-1BBL$^{-/-}$ mice such that mice succumbed to lethal viral challenge. These data support a role for 4-1BBL in memory CD8$^+$ T cell responses to LCMV.

Intraportal delivery of influenza virus results in a fairly strong immune response in the spleen with 7% of CD8 cells specific for the major CTL epitope NP366-374 at the peak of the response. However, influenza does not replicate extensively in the mouse. Thus, we did not observe increases in the size of the spleen of the influenza-infected mice. In contrast, LCMV replicates extensively in mice leading to significantly higher expansion of viral-specific CD8$^+$ T cells in the spleen than seen in influenza infection. For example, ~10$^7$ LCMV-NP396–404-specific CD8$^+$ T cells are seen in the spleen at day 8 following LCMV infection (20) whereas only 10$^6$ influenza-NP366-374-specific CD8$^+$ T cells are found in the spleen at day 7 following influenza infection (in this report).

These differences in the amount of viral replication and the tremendous increases in numbers of CD8$^+$ T cells during the response to LCMV might explain the differences in the kinetics of the effect of 4-1BBL that we see in this study compared with the studies of 4-1BBL mice (20). During the LCMV response, defects were observed at day 8 in both CD28$^{-/-}$ mice (30) and 4-1BBL$^{-/-}$ mice (20) although it is difficult to evaluate kinetic differences in the effects of these costimulatory pathways, as only the day 8 time point was reported. In the present study, we observed a clear segregation of the effects of CD28 vs 4-1BBL costimulation, where CD28$^{-/-}$ mice have defects much earlier in the response to infection than the 4-1BBL$^{-/-}$ mice. Furthermore, in the influenza model, the effects of CD28 were much more dramatic than the effects of 4-1BBL, whereas in the LCMV model the effects of CD28 were similar in magnitude to the effects of 4-1BBL, that is ~2-fold when converted to total numbers of tetramer-positive CD8$^+$ T cells per spleen (20, 30). We do not think our failure to detect an effect of 4-1BBL on primary T cell expansion is due to insensitivity of the assay or due to high Ag load. In the case of LCMV, the response was larger and yet both CD28$^{-/-}$ and 4-1BBL$^{-/-}$ mice showed defects at day 8 after infection. In our studies, we could readily detect a defect in the CD28$^{-/-}$ mice early in the response, but only detected the effect on the 4-1BBL$^{-/-}$ mice later in the response. The very large expansion of CD8$^+$ T cells due to extensive LCMV replication early during the infection may accelerate the timing of the effects of costimulation. In contrast, the more modest expansion of CD8$^+$ T cells during the immune response to influenza has allowed us to visualize a temporal segregation of the effects of the CD28 vs 4-1BB costimulatory pathways, with CD28 clearly acting very early in the response and 4-1BBL only impacting on T cell numbers much later in the response.

Members of the TNFR family, including 4-1BB are known to influence cell survival by activating the NF-xB pathway (31), which in turn can lead to up-regulation of Bcl-xL (32) as well as cellular inhibitors of apoptosis 1 and 2 (33). Upon aggregation of 4-1BB on a T cell, 4-1BB recruits TNFR-associated factors 1 and 2 which in turn links 4-1BB to the NF-xB pathway as well as the p38 and stress-activated protein kinase/c-Jun N-terminal kinase mitogen-activated protein kinase pathways (15, 31, 34–36). Both CD28 as well as members of the TNFR family, including OX40, influence Bcl-xL expression (37–39). Thus, it is conceivable that several pathways impact on regulation of T cell survival perhaps
acting to sustain or reinforce Bcl-x<sub>L</sub> expression as well as other survival signals. We used intracellular staining for Bcl-x<sub>L</sub> to try to detect differences in Bcl-x<sub>L</sub> expression on tetramer-positive influenza-specific T cells. However, the staining with anti-Bcl-x<sub>L</sub> was too weak to draw conclusions (data not shown). In addition to members of the Bcl-2 family, long-term T cell survival can also be regulated by cytokines. For example, pretreatment with IL-4 can affect long-term CD8 T cell survival (29). Furthermore, CD8 memory T cell survival has been shown to be dependent on IL-15 (40–42). Thus, another possible mechanism of 4-1BB-induced survival of the memory pool would be to influence the responsiveness of the memory T cell pool to cytokines.

Other TNFR family members have also been suggested to be important in sustaining T cell activation. For example, CD27−/− mice showed a reduced number of CD4 and CD8 T cells infiltrating the lungs during secondary influenza virus infection (43), although there was no decrease in CTL effector function as a result of the CD27 deficiency. OX40 has also been shown to affect the immune response to influenza virus (44). In this case, the effects of OX40 deficiency were limited to the CD4 T cell subset (44)−−44 with no detectable effect on the CD8 T cell response (44). Recently, it was demonstrated using in vitro analysis of TCR transgenic T cells lacking CD28 or OX40 that CD28 regulates Bcl-2 family levels at days 2−4 after activation and that OX40 is responsible for prolonging the life of anti-OX40-treated TCR transgenic T cells by regulating Bcl-x<sub>L</sub> and Bcl-2 later in the immune response. These data provide evidence for sequential action of CD28 and OX40 on T cell survival in vitro and are entirely consistent with our data on the temporal segregation of the effects of 4-1BB from those of CD28 in vivo. Thus, CD28 appears to be responsible for the first wave of survival signals and this can then be sustained by inducible costimulatory pathways such as OX40 or 4-1BB. It is likely that these survival signals are multifaceted and include anti-apoptotic as well as pro-survival signals. The precise molecular signals induced by each of the costimulatory pathways remains to be fully elucidated.

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References


