Endogenous 4-1BB Ligand Plays a Critical Role in Protection from Influenza-Induced Disease[1]

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A critical issue during severe respiratory infection is whether it is the virus or the host response that does the most damage. In this study, we show that endogenous 4-1BBL plays a critical role in protecting mice from severe effects of influenza disease. During mild respiratory influenza infection in which virus is rapidly cleared, the inducible costimulatory receptor 4-1BB is only transiently induced on lung T cells and 4-1BB ligand (4-1BBL) is completely dispensable for the initial CD8 T cell response and mouse survival. In contrast, during more severe respiratory influenza infection with prolonged viral load, 4-1BB expression on lung CD8 T cells is sustained, and 4-1BBL-deficient mice show decreased CD8 T cell accumulation in the lungs, decreased viral clearance, impaired lung function, and increased mortality. Transfer of an optimal number of naive Ag-specific T cells before infection protects wild-type but not 4-1BBL-deficient mice from an otherwise lethal dose of influenza virus. Transfer of T cells lacking the proapoptotic molecule Bim extends the lifespan of 4-1BBL-deficient mice by one to three days, suggesting that at least part of the role of 4-1BB/4-1BBL is to prolong effector cell survival long enough to clear virus. Intranasal delivery of 4-1BBL by recombinant adenovirus marginally improves survival of 4-1BBL-deficient mice at low dose, but exacerbates disease at high dose. These findings suggest a rationale for the evolutionary accumulation of inducible costimulatory molecules, thereby allowing the immune system to sustain the expression of molecules such as 4-1BB to a level commensurate with severity of infection. ***The Journal of Immunology,*** **2009, 182: 934–947.**

The CD8 T cells are important mediators of protective immunity to viruses (1). Indeed a strong CD8 T cell response to influenza offers the hope of cross-protective immunity to diverse influenza strains (2, 3). However, there is a concern that too strong an immune response in the lung will cause immune pathology and a worse outcome (4–6). Upon initial encounter with Ag, T cells commit to programmed expansion, which occurs independently of the continued presence of Ag (7, 8). How then does the immune system provide the appropriate level of T cell stimulation for a particular infection? T cells regulate their level of response by integrating signals from the Ag-specific receptor as well as from costimulatory, coinhibitory, and cytokine receptors which in turn influence the initiation, duration, and differentiation of the response (9–12). The initial activation of T cells requires costimulatory signals from CD28, expressed on resting T cells. In addition, during an on-going immune response, other costimulatory receptors and ligands are up-regulated on the T cells and APCs (13–15). The question arises as to why so many costimulatory receptor ligand pairs have accumulated in the mammalian immune system. Here we provide evidence that the expression of one such costimulatory receptor, 4-1BB, is sustained in response to severe as compared with mild respiratory infection with influenza virus, thereby allowing the immune system to maintain a level of CD8 T cell response appropriate for the severity of the infection.

The receptor 4-1BB (CD137) is a member of the TNFR family expressed on Ag-receptor activated T cells (14, 16–18). In vivo with nonreplicating immunogens, 4-1BB is rapidly and transiently up-regulated on T cells upon immunization, before the first cell division and concomitantly with the expression of the early activation marker CD69 (19). Evidence that 4-1BB can play a role early in the response comes from systemic administration of agonist anti-4-1BB Abs, which can enhance initial antitumor and antiviral T cell responses with greater effects on CD8 compared with CD4 T cells (20–24). However, studies in 4-1BB ligand (4-1BBL)[4]-deficient mice showed that 4-1BBL was dispensable for primary responses to nonreplicating Ags or to influenza virus delivered via a minimally infectious systemic route (19, 25). Under these conditions, 4-1BBL was found to control the magnitude of the recall response to influenza virus (25), later attributed to a role for 4-1BBL in maintaining CD8 T cell survival in the weeks after initial infection (26). In contrast, with stronger immune responses such as skin allograft rejection or lymphocytic choriomeningitis virus infection (27, 28), the effect of 4-1BBL was detected during priming. Thus, we hypothesized that the requirement for 4-1BBL during T cell priming might depend on the severity of the disease model.

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[4] Abbreviations used in this paper: 4-1BBL, 4-1BB ligand; FMO, fluorescence minus one; HAU, hemagglutinating units; i.n., intranasal; AdV, adenovirus 5; MLN, mediastinal lymph node; WT, wild type.

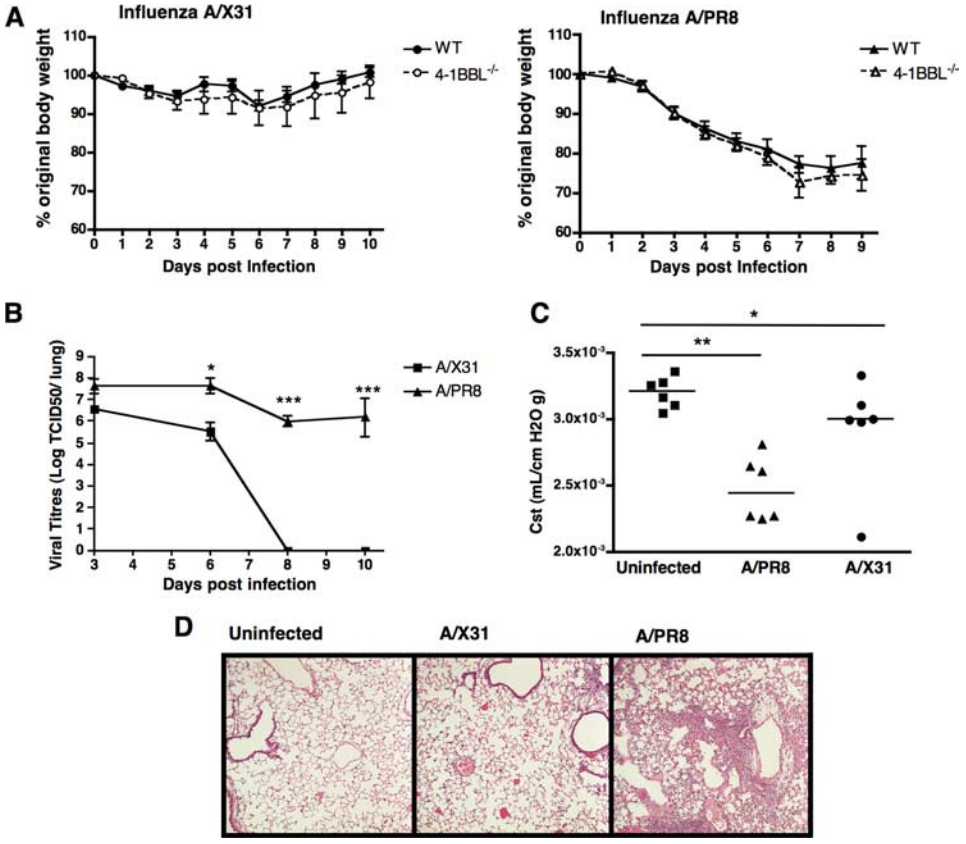

FIGURE 1. Disease outcomes in mild vs severe respiratory influenza infection. WT or 4-1BBL$^{-/-}$ mice were infected i.n. with 5 HAU of Influenza A/X31 or 2.5 HAU of influenza A/PR8. *A*, Weight loss of the infected mice was monitored daily. Results are representative of three experiments with $n = 4–6$ mice per group. *B*, Lung homogenates were analyzed for viral load at different times after infection with $n = 2–5$ WT mice per group per time point. *C*, Lung compliance (volume/pressure) was measured at day 6 following PR8 or X31 infection of WT mice. Bar indicates median levels, and data are shown for individual mice representative of two such experiments. *D*, H&E analysis of WT lung sections at day 8 postinfection. Results in *A* and *B* are shown as mean ± SEM. Results in *B* and *C* were analyzed by one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

To test the requirement for 4-1BBL as a function of disease severity, we compared immune responses and disease outcome in wild-type (WT) and 4-1BBL-deficient mice after respiratory infection with two well-studied strains of influenza virus that share the same CD8 T cell epitopes but differ in their virulence in mice (described in Ref. 29 and references therein). The results show that 4-1BBL is completely dispensable for the primary CD8 T cell response and mouse survival following a mild respiratory infection with influenza A/HKx31 or in response to i.p. infection with influenza A/PR8. In contrast, following intranasal (i.n.) infection with the more virulent A/PR8, 4-1BBL-deficient mice have a worse disease outcome, as measured by loss of lung compliance, impaired viral clearance, and increased mortality. This poor outcome correlated with a decreased CD8 T cell response to influenza virus in the lungs of 4-1BBL-deficient mice. In a TCR transgenic T cell model, transfer of an optimal number of naive Ag-specific T cells before infection protects WT but not 4-1BBL-deficient mice from an otherwise lethal dose of influenza virus. T cells lacking the proapoptotic molecule Bim showed modestly improved protection following adoptive transfer into 4-1BBL-deficient mice, suggesting that at least part of the role of 4-1BB/4-1BBL is to prolong effector cell survival long enough to clear virus. The i.n. delivery of a low dose of 4-1BBL-adenovirus 5 (4-1BBL-AdV) marginally prolonged the survival of 4-1BBL-deficient mice, but impaired the survival of the WT mice. Moreover, a high dose of 4-1BBL-AdV impairs survival of both WT and 4-1BBL-deficient mice. These findings suggest that the endogenous level of 4-1BB and its ligand are tightly controlled to provide optimal protection while avoiding immunopathology.

Materials and Methods

Mice

WT (C57BL/6) breeder pairs were obtained from Charles River Laboratories. The 4-1BBL-deficient (4-1BBL$^{-/-}$) mice on the C57BL/6 background ($n = 9$) (27) (obtained under a materials transfer agreement from Immunex, now Amgen) were bred under specific pathogen-free conditions at the University of Toronto. CD45.1 congenic mice and OT-I mice were obtained from The Jackson Laboratory and crossed to generate CD45.1 OT-I mice. These mice were also crossed with Bim$^{-/-}$ mice obtained from P. Ohashi (Ontario Cancer Institute, Ontario, Canada). All mouse experiments were approved by the University of Toronto animal care committee in accordance with the regulations of the Canadian Council on animal care.

Viruses, infections, and titers

Influenza A/PR8-OVA (30) was obtained from P. Thomas and P. Doherty (St. Jude's Hospital, Memphis, TN). Influenza A/PR8, influenza A/PR8-OVA, and influenza A/HKx31 were grown in eggs and their tissue culture infectious dose determined by infection of MDCK cells (31). For i.n. infection, age- and sex-matched mice of between 6 and 10 wk of age were anesthetized with isofluorane. For primary i.n. infection with influenza A/PR8, a dose of 0.25–5 hemagglutinating units (HAU, equivalent to $\sim 10^3$—2×10^4 $TCID_{50}$) was given, as indicated in the experiments. For PR8-OVA, a dose of $2.5–5 \times 10^5$ $TCID_{50}$ was used. Mice were monitored closely and sacrificed when moribund or at the times indicated in the experiment. For i.n. infection with A/X31, a dose of 5 HAU (equivalent to 3.86×10^4 $TCID_{50}$) was used. For i.p. infection, mice were infected with 200 HAU of influenza PR8. Body weights of infected mice were recorded daily for up to 16 days to monitor the progression of disease and recovery from infection. For analysis of lung T cells, lungs were perfused with 10 ml of ice-cold PBS and lymphocytes from individual mice were then enriched by isolation over an 80/40% Percoll gradient. For viral clearance assays, lungs were excised from the animal at various time points post i.n. infection with 0.25–2.5 HAU of influenza A/PR8 or 5 HAU of influenza A/X31. After weighing, lungs were homogenized in RPMI 1640 medium (1 g of lung tissue/10 ml). Supernatant was obtained and stored at −70°C after centrifugation at $1200 \times g$ for 20 min at 4°C. $TCID_{50}$ was determined by the MDCK assay with the Reed and Muench technique as previously described (31).

Adenovirus delivery of 4-1BBL

A replication-defective adenovirus 5 (AdV) vector expressing full length murine 4-1BBL was generated by the two plasmid rescue method (32), the virus was purified and the titer was determined by plaque assay, as described for human 4-1BBL-AdV (33). The i.n. delivery was performed

using 10^5–10^9 PFU of adenovirus diluted in PBS, as indicated in each experiment.

Lung histology

Mice were euthanized by CO_2 inhalation and immediately a cannula was placed in the trachea and secured with a suture. The lungs were inflated with 10% formalin at a constant inflation pressure of 20 cm H_2O and then the tracheal opening was closed with the suture. Lungs were subsequently removed and placed in 10% formalin for 24 h. Paraffin embedding, sectioning, and H&E staining were performed at the Toronto Centre for Phenogenomics (Toronto, Ontario, Canada).

Measurement of lung function

For measurements of lung compliance, mice were sedated, paralyzed, and a tracheostomy performed. A cannula was inserted and the mice were attached to a ventilator equipped with a pressure and volume (flow) transducer (flexiVent; SciReq). Static compliance measurements were obtained from dynamic pressure volume curves using the Salazar-Knowles equation (34).

T cell isolation and adoptive transfers

OT-I T cells were purified from spleens of uninfected mice using a negative selection mouse CD8 T cell enrichment kit (StemCell Technologies), and injected i.v. into mice 24 h before infection with influenza A/PR8-OVA.

Flow cytometry

Analysis of influenza $NP_{366-374}$-specific CD8 T cells using MHC tetramers as well as intracellular cytokine staining following a 6 h restimulation was conducted as previously described (25). Uninfected mice were used as negative controls for $D^b/NP_{366-374}$ tetramers staining. Isotype control or fluorescence minus one (FMO) control were used as negative control for cytokine staining. For subdominant epitopes, cells were stimulated with 10 μM of the relevant peptide for 8 h, with GolgiStop (BD Pharmingen) added for the last 5 h of the stimulation. Congenically marked OT-I TCR transgenic cells were tracked by using PE or allophycocyanin anti-mouse CD45.1 (eBioscience) in conjunction with PerCp anti-mouse (BD Biosciences) or PE anti-mouse CD8. For detection of degranulation, 5 μg/ml FITC anti-CD107a (eBioscience) was added to the restimulation culture containing 1 μM $OVA_{257-264}$ peptide and GolgiStop. Cells were incubated for 6 h as described. FMO was used as a negative control for analysis of CD107a level. Other Abs used in this study included PE anti-mouse IFN-γ, FITC anti-mouse NK1.1, FITC Annexin V and customized AF488 anti-CD49a (BD Biosciences), PE anti-mouse CD3, PE anti-mouse-4-1BB, FITC anti-$\gamma\delta$ TCR, PE anti-B220, allophycocyanin anti-CD11c, PE-Cy5 anti-mouse TCRβ, PE-Cy5 anti-mouse CD19, allophycocyanin anti-CD44, PE anti-CD11a, and biotinylated anti-mouse CD45.2 (eBioscience)and FITC anti-PDCA-1 (Miltenyi Biotec). Samples were analyzed using a FACSCalibur (BD Biosciences) and FlowJo software (TreeStar). The 10^9 PFU adenovirus control-infected 4-1BBL-deficient mouse was used as a negative control for analysis of 4-1BBL expression following 4-1BBL adenovirus delivery.

Cytotoxicity assay

CTL activity was measured in a direct ex vivo CTL assay as previously described (20). The percentage of specific lysis was calculated using the following equation: (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release) × 100.

Analysis of 4-1BBL expression by PCR

Organs were obtained from infected mice on day 0, 3, 6, and 8 postinfluenza infection. Lungs were homogenized in 2 ml TRIzol (Invitrogen) and cell suspensions of spleens and mediastinal lymph node (MLN) were resuspended in 1 ml of TRIzol, followed by mRNA extraction and purification using RNeasy Mini kit (Qiagen). Reverse primers against 4-1BBL (5′-GCTTGGCGAACACAGGAG-3′) and β-actin (5′-AAGAAGGAAGG CTGGAAA-3′) and purified RNA were used for the reverse transcription reaction (Invitrogen). PCR of the cDNA was done using forward primers 5′-CTTGATGTGGAGGATACC-3′ for 4-1BBL and 5′-GGGAATGGGTC AGAAGGA-3′ for β-actin, in conjunction with the reverse primers described. Thirty-six cycles were performed for the amplification of 4-1BBL cDNA with an annealing temperature of 50.3°C. Thirty cycles were performed with β-actin with the annealing temperature of 52°C. Serial dilutions of the cDNA templates were done to ensure that the amplification products of 4-1BBL and β-actin did not reach saturation (data not shown). The intensity of the 4-1BBL band was normalized to the β-actin level using Quantity One software (Bio-Rad).

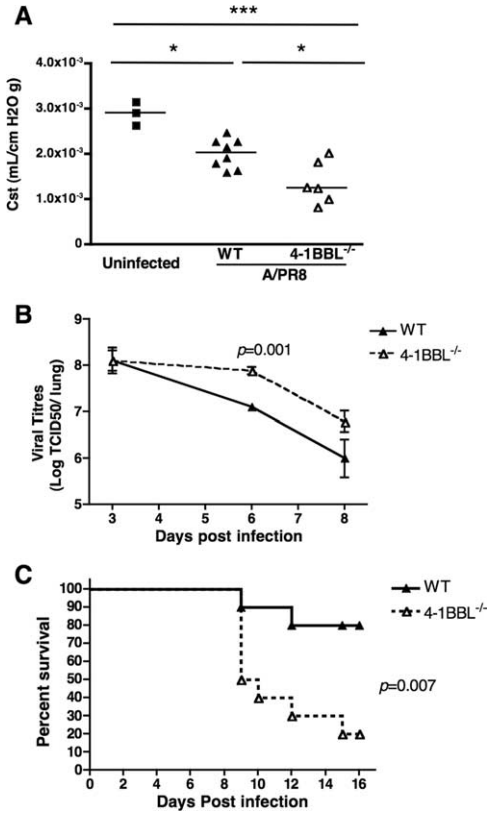

FIGURE 2. 4-1BBL plays a critical role in mouse survival and viral clearance following severe respiratory infection in mice. WT or 4-1BBL$^{-/-}$ mice were infected i.n. with 2.5 HAU of influenza A/PR8 and monitored for lung function (*A*) on day 6 (data are pooled from two experiments with each symbol representing one mouse) and viral load (*B*) at days 3–8 (data shown are the average of $n = 3$–6 mice per group per time point and representative of two experiments). Additional experiments at 0.25 HAU also showed a similar 5-fold increase in viral load at day 6–8 in 4-1BBL-deficient compared with WT mice (data not shown). *C*, WT or 4-1BBL$^{-/-}$ mice ($n = 10$ per genotype) were infected i.n. with 0.25 HAU of PR8 and monitored for survival, with data pooled from two different experiments. Additional experiments using viral doses of 0.75 and 0.08 HAU also showed a survival advantage of WT as compared with 4-1BBL-deficient mice ($n = 4$–5 mice per group, data not shown). Medians are shown in *A* and mean ± SEM is shown in *B*. Results in *A* were analyzed by one-way ANOVA. ∗, $p < 0.05$ and ∗∗∗, $p < 0.001$. An unpaired *t* test was used to compare WT and 4-1BBL-deficient mice at each individual time point. Log-rank test was used in *C*.

Statistical analysis

Where indicated, for comparison of two values, *p* values were obtained using the Student's *t* test (unpaired, two tailed, 95% confidence interval). One-way ANOVA was used to compare multiple samples, and statistically significant differences were indicated. A log-rank test was used for analysis of the survival curve (95% confidence interval).

Results

Differential disease induction following i.n. infection with Influenza A/HKx31 or A/PR8 virus

Influenza A/PR8 (H1N1, PR8) causes an acute respiratory infection in mice that can be lethal (35). Influenza A/HKx31 (H3N2, X31) is a recombinant virus containing the hemagglutinin (H) and neuraminidase genes (N) from the 1968 Hong Kong strain of influenza virus but the internal viral proteins, including the major

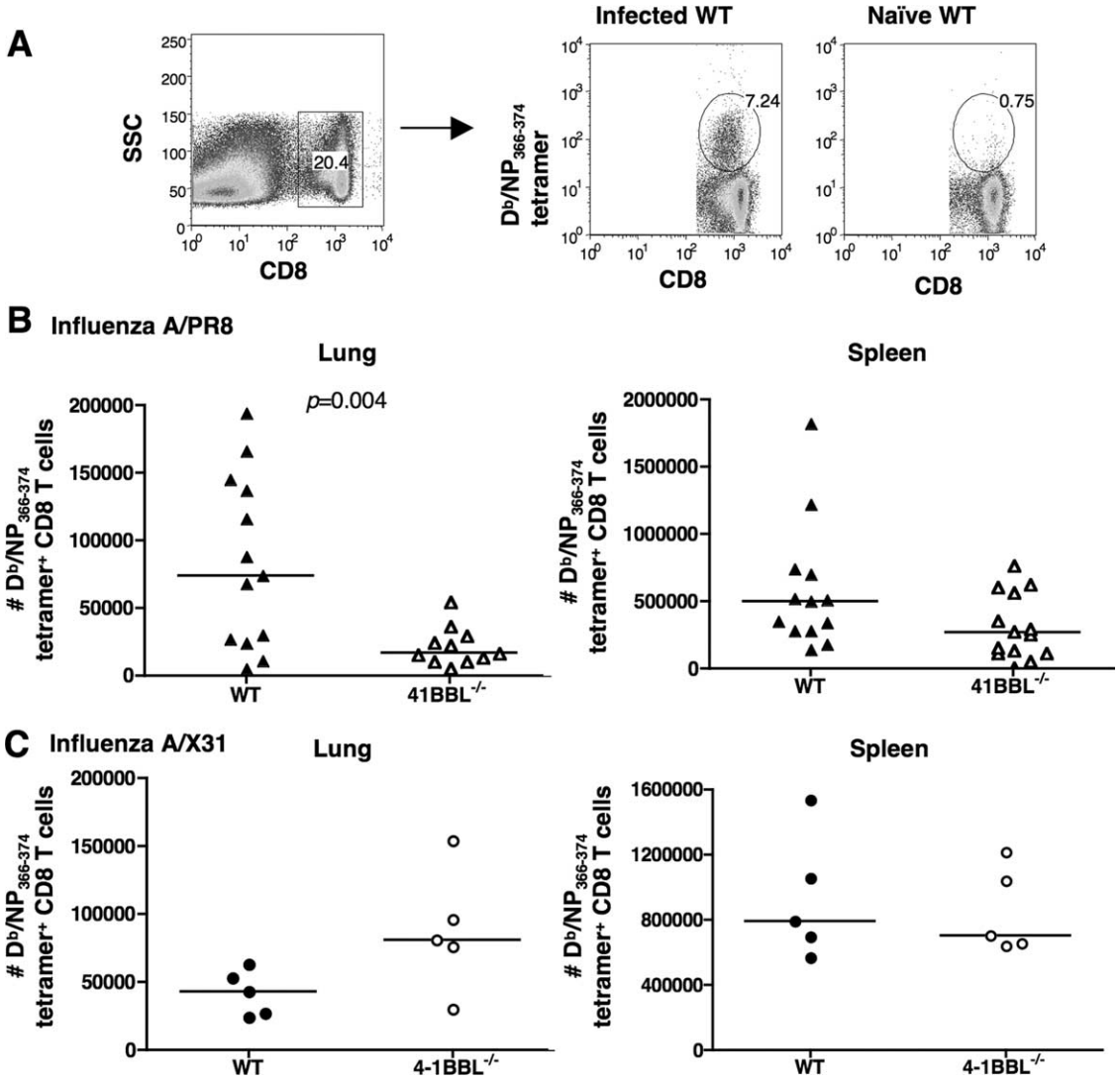

FIGURE 3. Analysis of Ag-specific CD8 T cells in WT and 4-1BBL$^{-/-}$ mice following i.n. infection with A/PR8 or A/X31. Mice were i.n. infected with 2.5 HAU of PR8 or 5 HAU of influenza A/X31 as in Fig. 1. CD8 T cell frequency and number in different organs were analyzed at day 9–10 postinfection using $D^b/NP_{366-374}$ tetramers. *A*, Representative staining for tetramer-positive cells in uninfected or PR8-infected mice. *B*, PR8 data pooled from three independent experiments, with each data symbol representing an individual animal. *C*, X31 data are representative of three experiments. Bar indicates median levels, and results were analyzed by the unpaired *t* test.

cytotoxic T cell epitopes, are from the PR8 strain (2). Infection of mice with the X31 virus induces a mild respiratory infection (36) that protects the mice from the more virulent PR8 virus (2, 37) (see Supplemental Fig. S1A).[5] To test the hypothesis that dependence on 4-1BBL is influenced by the severity of the infection, we first confirmed that primary infection with PR8 and X31 differ in the severity of disease. The i.n. infection with X31 causes mice to lose up to 10% of their body weight and then fully recover with no detectable viral load in the lungs by day 8 postinfection (Fig. 1, *A* and *B*). Increasing the viral innoculum by 70-fold still resulted in no more than 15% weight loss and full recovery of the mice (data not shown). In contrast, i.n. infection with PR8 results in loss of up to 30% of the body weight of the mice. Most of the PR8-infected mice were moribund and were euthanized on day 9 postinfection, and those that survived until day 10 failed to control the virus (Fig. 1, *A* and *B*).

Noninvasive estimates of lung function have been shown to correlate with inflammation in other viral models (38). To analyze the effect of infection on lung function in the two models, mice were sedated and tracheostomized at day 6 postinfection, the time point at which weight loss reached a maximum for the X31 infection. Compared with uninfected mice, infection with either virus caused a loss of lung compliance with more substantial changes noted in the PR8 infection (Fig. 1*C*). Consistent with the physical readouts shown, H&E staining of infected lung sections showed a more extensive infiltrate in the PR8- compared with X31-infected lungs (Fig. 1*D*). These studies confirm, using the measurement of lung function as an objective measure of influenza disease, that influenza PR8 causes more severe influenza disease than influenza X31.

Effect of 4-1BBL on disease outcome during severe influenza virus infection of mice

The 4-1BBL deficiency had no effect on weight loss in response to either X31 or PR8 infection (Fig. 1*A*), likely because this initial weight loss is largely due to innate cytokine production (39). However, PR8-infected 4-1BBL-deficient mice had reduced lung compliance compared with infected WT mice suggesting a role for 4-1BBL in disease outcome (Fig. 2*A*). This impaired lung function

[5] The online version of this article contains supplemental material.

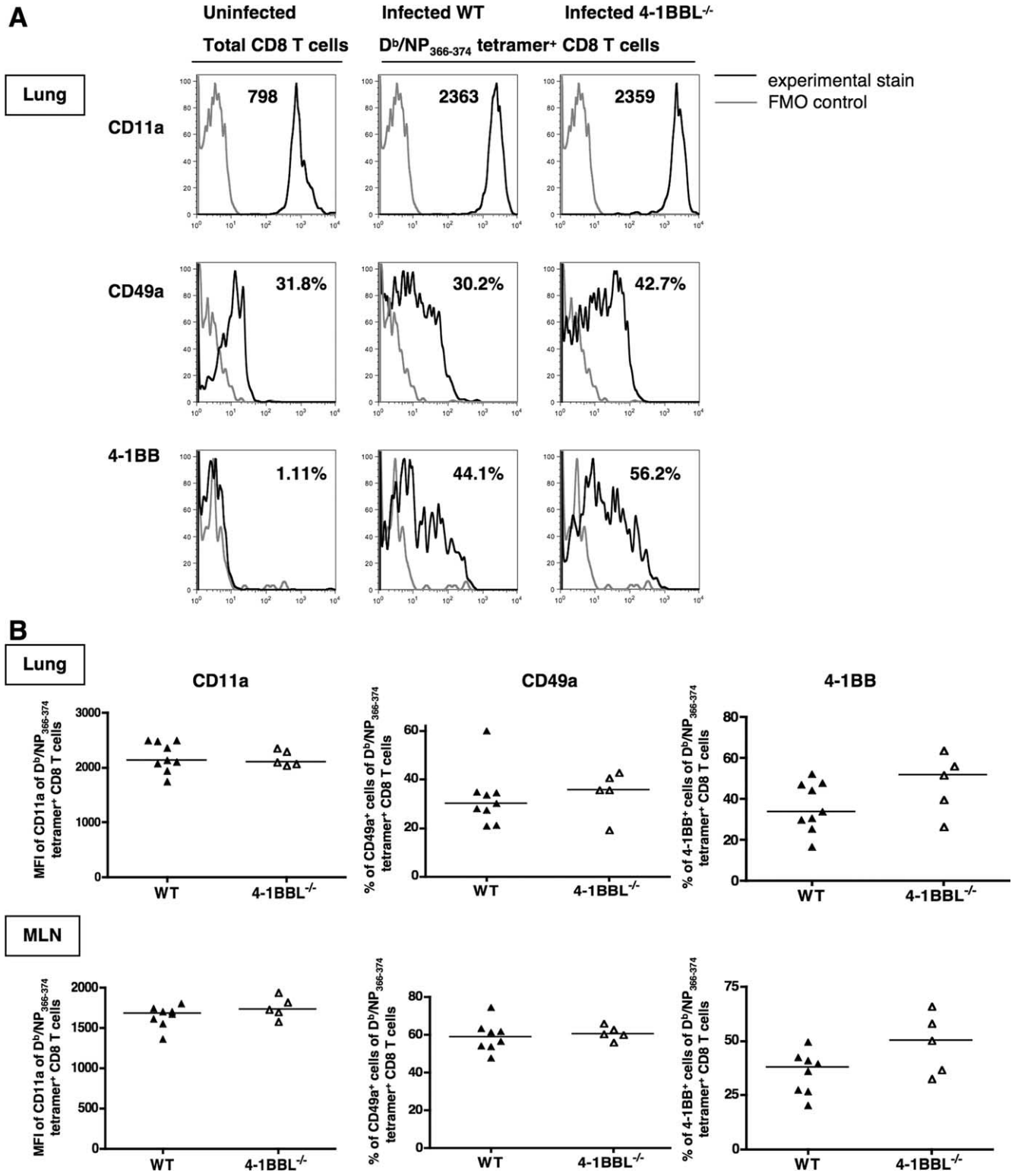

FIGURE 4. Analysis of LFA-1, VLA-1, and 4-1BB expression in WT and 4-1BBL-deficient mice following influenza A/PR8 infection. Mice were infected i.n. with 2.5 HAU of influenza A/PR8 as in Fig. 1. CD11a, CD49a, and 4-1BB expression on $NP_{366-374}$-specific CD8 T cells in infected mice or total CD8 T cells in uninfected mice was analyzed on day 8 postinfection. *A*, The median fluorescence intensity (MFI) of CD11a, the percentage of $CD49a^+$, and the percentage of $4\text{-}1BB^+$ cells are shown, respectively. *B*, Summary plots of the CD11a median fluorescence intensity, percentage of $CD49a^+$, and percentage of $4\text{-}1BB^+$ on $NP_{366-374}$-specific CD8 T cells in the lungs and MLN are shown. Bar indicates median levels, and no significant difference was obtained with unpaired *t* test. Each data point represents an individual mouse, with a total of $n = 5$–9 mice per group.

correlated with a 0.7 log higher viral load in 4-1BBL-deficient as compared with WT mice at day 6 and 8 postinfection ($p = 0.001$ at day 6) (Fig. 2*B*).

To test the effect of 4-1BBL on mouse survival, we lowered the dose of virus by 10-fold and under these conditions, 80% of WT mice survived the respiratory PR8 infection and cleared the virus

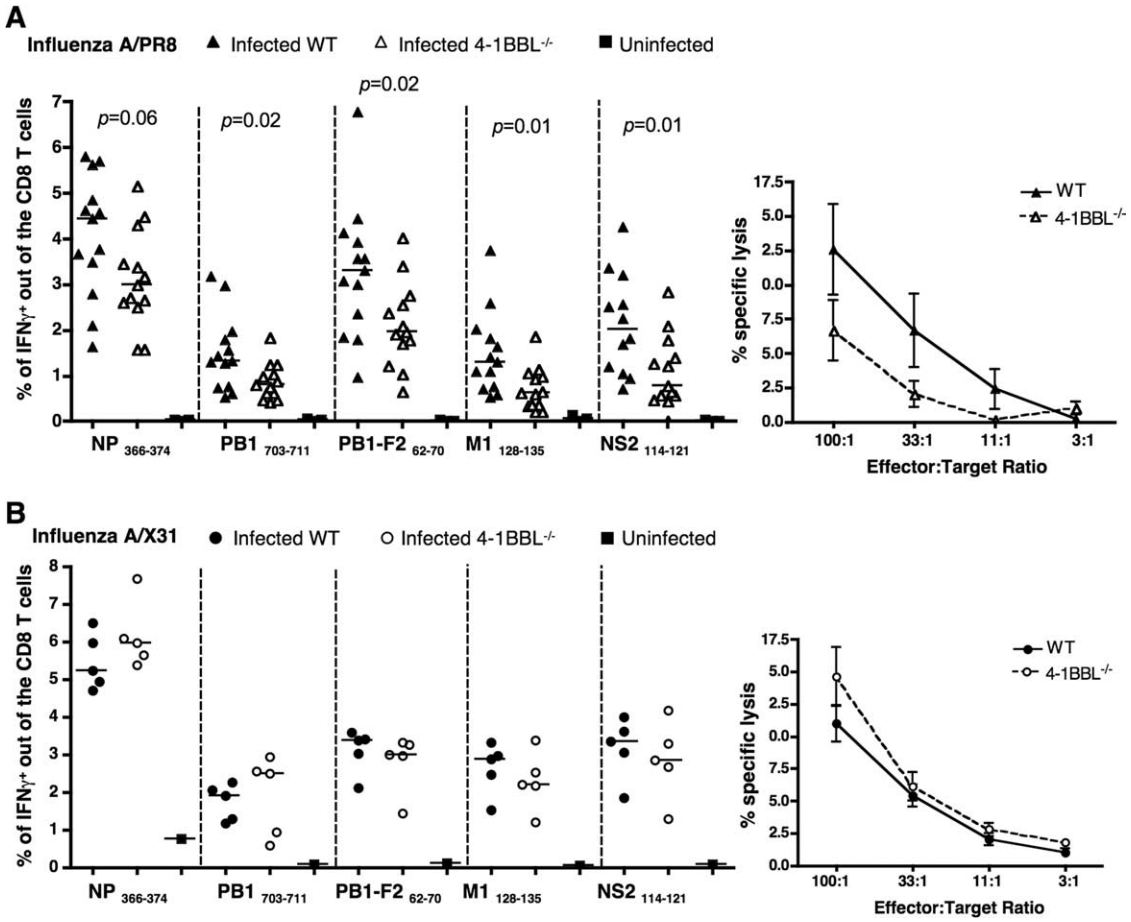

FIGURE 5. Analysis of response to subdominant epitopes in WT and 4-1BBL-deficient mice. Mice were i.n. infected with 2.5 HAU of influenza A/PR8 (*A*) or 5 HAU of influenza A/X31 (*B*) as in Fig. 1. Results are shown for splenic T cells restimulated for 6 h with the indicated peptide. Each data point represents an individual mouse (*left*). Bar indicates median levels. Results were analyzed by the unpaired *t* test. T cell cytotoxicity was analyzed in a direct ex vivo ^{51}Cr release assay with $NP_{366-374}$-pulsed EL4 target cells (*right*) as described in *Materials and Methods*. Results represent mean ± SEM of n = 5–6 mice per group, representative of two experiments.

by day 8–10 postinfection. In contrast, 80% of 4-1BBL-deficient mice succumbed to the PR8 infection ($p = 0.007$) (Fig. 2*C*), and viral load was five times greater in the 4-1BBL-deficient mice compared with WT mice by day 8 postinfection ($p = 0.06$, data not shown).

To determine whether the defects in lung function, viral clearance and mouse survival could be correlated with the immune response, we analyzed different immune cell subsets in the mice following infection. Following i.n. infection with PR8, similar numbers of CD4 T cells, γδ T cells, NK, NKT, $CD11c^{high}$, $CD11c^{int}$ (dendritic cells and monocytes), and plasmacytoid dendritic cells were recovered from the lungs of WT and 4-1BBL-deficient mice (see Supplemental Fig. S2).[5] However, Ag-specific CD8 T cell numbers were significantly reduced in the lungs of 4-1BBL-deficient compared with WT mice on day 9 postinfection with PR8 (Fig. 3*B*), with a similar trend in the spleen (Fig. 3*B*), but no detectable difference in MLN (data not shown). We focused on day 9 postinfection because at earlier time points, the number of Ag-specific CD8 T cells in the lungs was low and therefore difficult to quantify accurately. However, the decrease in Ag-specific T cell numbers observed is unlikely to be due to a kinetic effect, as a similar number of Ag-specific CD8 T cells were recovered in the draining lymph node (MLN) of WT and 4-1BBL-deficient mice at day 6 postinfection (~10,000–12,000 ± 3000 $NP_{366-374}$-specific

Table I. *Influenza epitopes studied*

Viral Strain[a]	Epitope	Sequence	MHC Restriction	EC_{50} (pM)[b]	Reference for Epitope
A PR8	NP 366–374	ASNENMETM	D^b	5.7	69
A PR8	PB1 F2 62–70	LSLRNPILV	D^b	n.d.	70
A PR8	M1 128–135	MGLIYNRM	K^b	3400	71
A PR8	NS2 114–121	RTFSFQLI	K^b	200	71
A PR8	PB1 703–711	SSYRRPVGI	K^b	100	72

[a] These epitopes are also shared by the influenza A X31 virus (reviewed in Ref. 2). PB1 F2, M1, NS2, and PB1 703 epitopes are subdominant during the primary response to influenza A PR8.

[b] EC_{50} for CTL assays were taken from Ref. 2 or from the original reference for those not included in this reference. PB1 F2 is in a second reading frame and has been compared for binding D^b in an RMA-S binding assay (similar to $NP_{366-374}$ in MHC class I binding affinity).

n.d., Not determined, similar to NP_{366} in binding affinity for D^b.

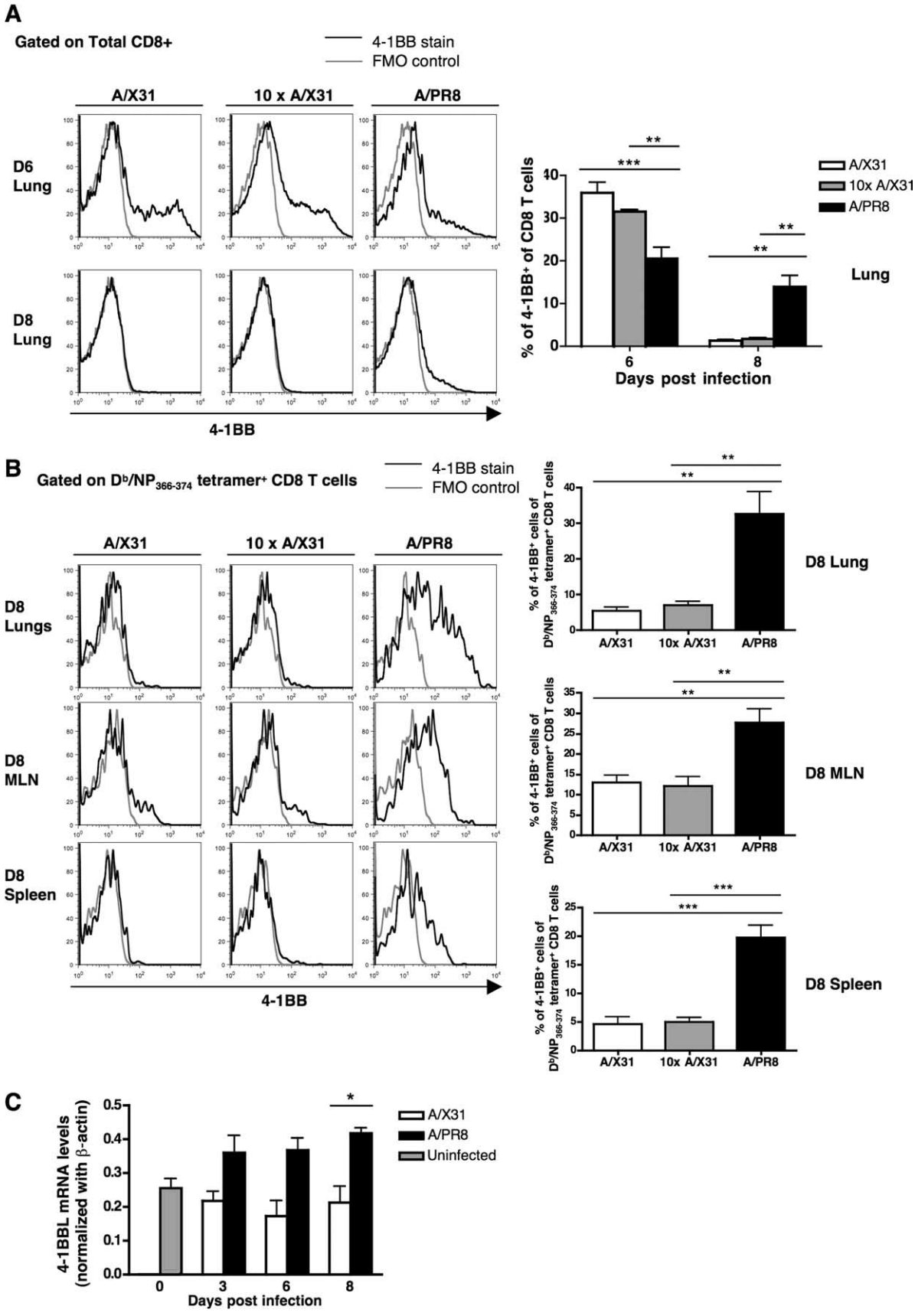

FIGURE 6. Analysis of 4-1BB and 4-1BBL expression in uninfected, X31-infected, and PR8-infected mice. Organs were obtained from mice infected with 5 or 50 HAU (10X) of X31 and 2.5 HAU of PR8 at day 6 or 8 postinfection. Surface expression of 4-1BB on total CD8 T cells (*A*) or on $NP_{366-374}$-specific CD8 T cells (*B*) was analyzed by flow cytometry. *A*, Representative FMO control staining (light gray histogram) and anti-4-1BB staining (black line histogram) are shown gated on total T cells. Summary plots of the lungs on day 6 and 8 are shown on the *right*. *B*, Representative data show 4-1BB expression on $NP_{366-374}$-specific CD8 T cells. Summary (*right*) shows a percentage of total $NP_{366-374}$-specific CD8 T cells staining for 4-1BB

cells, $p = 0.62$). Thus, PR8-infected 4-1BBL-deficient mice show decreased accumulation of Ag-specific CD8 T cells in the lungs compared with PR8-infected WT mice.

In contrast to the results with PR8 virus, i.n. infection with the X31 virus induced a similar frequency and number of CD8 T cells in the lungs, spleen, MLN, and bronchoalveolar lavage of WT and 4-1BBL-deficient mice, measured at the peak of the primary response (day 10) (Fig. 3*C* and data not shown). Similar results were previously reported for i.p. infection with X31 and were not due to a kinetic effect (25). These results suggest a differential role for 4-1BBL in primary Ag-dependent CD8 T cell responses to severe vs mild respiratory influenza infection.

The integrins LFA-1 and VLA-1 are up-regulated upon T cell activation in the lymph node and are important in the recruitment or retention of CD8 T cells in the lung during influenza infection (29, 40). Thus it was possible that a decreased number of CD8 T cells in the lung reflected incomplete activation leading to decreased trafficking or retention of T cells in the lung. Following PR8 infection, $NP_{366-374}$-specific T cells in lymph node and lung show evidence of up-regulation of CD11a (the α subunit of the LFA-1 integrin) to a similar extent in WT and 4-1BBL-deficient mice (Fig. 4*A*). A proportion of lung CD8 T cells express CD49a, the α subunit of VLA-1, independently of infection. Upon infection, the fraction of tetramer-positive T cells staining with CD49a was similar in WT and 4-1BBL-deficient mice (Fig. 4). The increase in the proportion of the $CD49a^{high}$ population in the 4-1BBL-deficient mice seen in Fig. 4*A* did not result in a statistically significant difference in the overall mean fluorescence intensity or the percentage staining when compared for several mice (Fig. 4*B* and data not shown). This increase in $CD49a^{high}$ cells might reflect that the 4-1BBL-deficient mice have a higher viral load than WT mice at this time point, resulting in more Ag-dependent activation.

The receptor 4-1BB is inducible on T cells by Ag-receptor signaling and therefore is a useful measure of the activation state of the cells. We therefore measured the level of 4-1BB on lung, draining lymph node, and splenic tetramer-positive T cells from WT and 4-1BBL-deficient mice following PR8 infection. The results show that 4-1BB levels per T cell are similar, or if anything slightly higher on Ag-specific T cells from 4-1BBL-deficient infected mice (Fig. 4 and data not shown for spleen). We also monitored CD44 levels on $tetramer^+$ T cells from PR8-infected WT and 4-1BBL-deficient mice and again found no difference in expression levels in lung, draining lymph node, and spleen (data not shown). Taken together, the data suggest that decreased accumulation of CD8 T cells in the lungs of 4-1BBL-deficient mice is not due to an impairment of their expression of activation and adhesion molecules.

Sensitivity to endogenous 4-1BBL is independent of epitope studied

The proportion of IFN-γ-producing cells was also reduced in 4-1BBL-deficient compared with WT mice for several different CD8 T cell epitopes following PR8 infection, with no defects observed in the X31-infected 4-1BBL-deficient mice (Fig. 5). Both dominant and subdominant epitopes required 4-1BBL for maximal responses in PR8-infected but not in X31-infected mice (Fig. 5). For several of these subdominant epitopes, T cell responses are of lower avidity (Table I), arguing that it is not the avidity of the T cell response but rather the severity of infection that influences the requirement for 4-1BBL in this CD8 T cell response. In support of this hypothesis, when PR8 was delivered by the less infectious i.p. route, which led to minimal viral replication and minimal weight loss, the CD8 T cell response was found to be 4-1BBL-independent (see Supplemental Fig. S3).[5] These results show that 4-1BBL is important for controlling T cell numbers during respiratory PR8 but not during X31 infection, independently of epitope studied.

CD8 effector function appeared to mirror the proportion of T cells present in the spleen, with a trend toward lower CTL killing in PR8-infected mice (Fig. 5*A*). Thus similar to the results with activation and adhesion molecules, 4-1BBL does not appear to affect the effector function per cell, but rather the number of cells recovered.

4-1BBL is dispensable for programming T cells for recall responses

To further establish that 4-1BBL was dispensable for CD8 T cell priming in response to influenza X31, we isolated T cells from X31-primed WT and 4-1BBL-deficient mice at 6 wk postinfection. Following transfer of a mixture of equal numbers of tetramer-positive cells into a naive WT mouse and rechallenge with X31, CD8 T cells primed in 4-1BBL-deficient mice were equally capable of re-expanding as compared with CD8 T cells primed in WT mice (see Supplemental Fig. S4).[5] In contrast, 4-1BBL is required for maximal recall responses to PR8 challenge following priming with X31 delivered i.n. (see Supplemental Fig. S1).[5] Similar results were obtained previously using the i.p. infection model, where the defective recall was attributed to a failure to maintain CD8 T cell numbers after Ag clearance (25, 26).

Similarly, when CD8 T cells were primed by Influenza PR8 infection of 4-1BBL-deficient mice, upon transfer to a WT host, T cells were equally capable on a per cell basis of secondary expansion to viral challenge as cells primed in a WT host (see Supplemental Fig. S4).[5] Thus, during i.n. or i.p. infection with influenza A/PR8 or A/X31, 4-1BBL appears to affect Ag-specific CD8 T cell numbers but is not required for "programming" the T cells for recall expansion.

Differential expression of 4-1BB on T cells in lungs of X31- vs PR8-infected mice

We next asked why 4-1BBL is critical during infection with the more virulent PR8 virus but not with the X31 virus. As 4-1BB is normally induced with Ag receptor signaling in T cells (41), we reasoned that the delayed viral clearance in the PR8-infected mice might result in prolonged 4-1BB expression, allowing 4-1BB to contribute to the CD8 T cell response and viral clearance during the later stages of viral infection. In the lungs, 4-1BB was detectable on total CD8 T cells on day 6 following X31 or PR8 infection (Fig. 6*A*), the time at which Ag-specific CD8 T cells first begin to accumulate in the lung (42). However, by day 8 postinfection, when Ag-specific responses were near maximal, 4-1BB expression was consistently observed on CD8 T cells of PR8-infected mice,

in the lungs of X31- or PR8-infected mice with $n = 3$–4 mice per group per time point. Mean ± SEM are shown in *A* and *B*, and results were analyzed by one-way ANOVA in $n = 4$ mice per group. **, $p < 0.01$ and ***, $p < 0.001$. Similar results at 5 HAU of X31 and 2.5 HAU of PR8 were obtained with another independent experiment with $n = 3$–5 mice per group per time point. *C*, mRNA was isolated from lungs of uninfected, X31-infected, or PR8-infected WT mice and 4-1BBL levels determined by reverse transcriptase PCR. Levels of 4-1BBL message were normalized to β-actin levels. Seven uninfected and three infected mice were used per group per time point. Two other independent experiments show similar results with the infected mice. Mean ± SEM is shown, and results were analyzed by one-way ANOVA. *, $p < 0.05$.

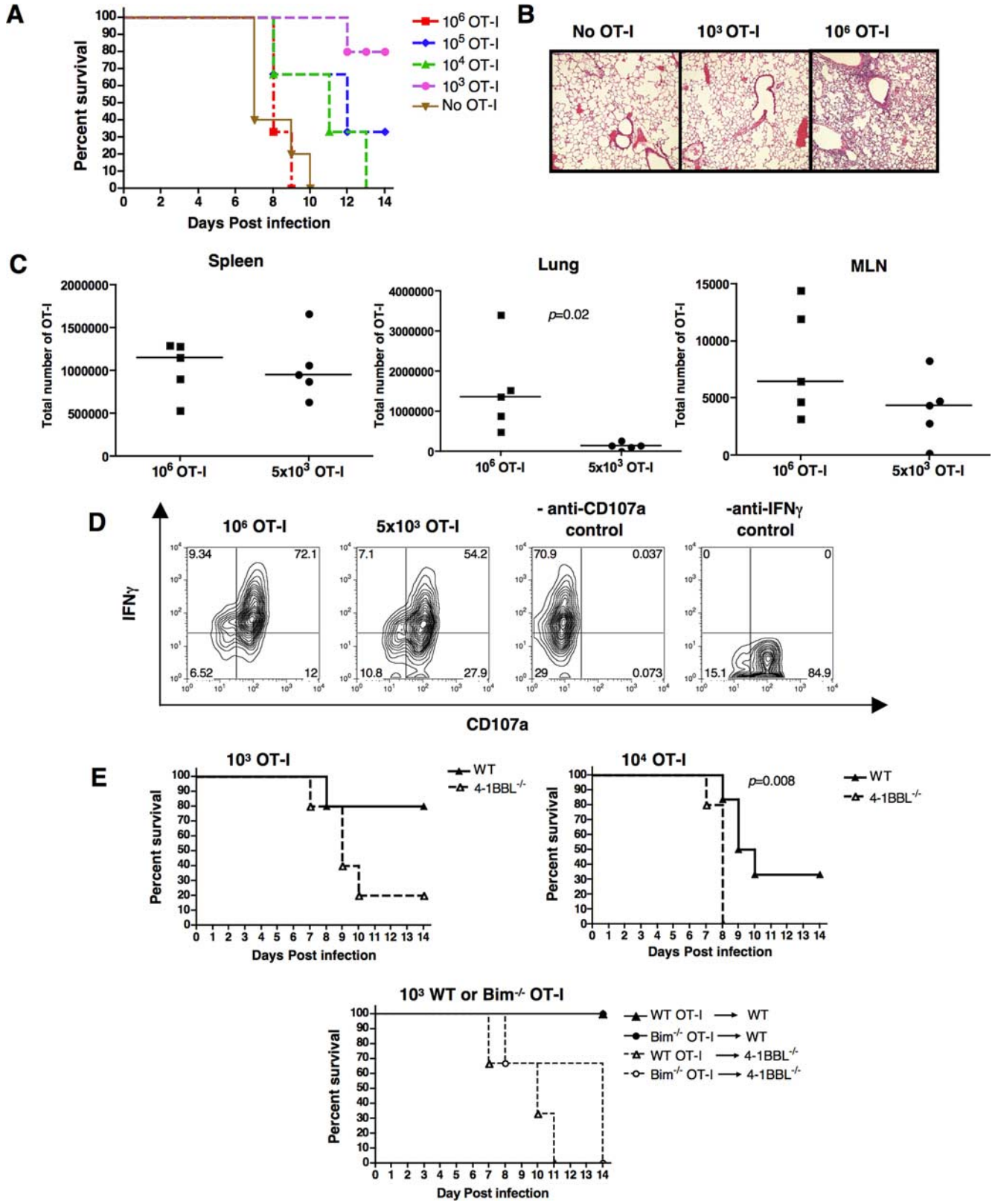

FIGURE 7. Protection of WT but not 4-1BBL$^{-/-}$ mice from lethal influenza infection by adoptive transfer of optimal numbers of naive Ag-specific T cells. Purified naive CD45.1 OT-I T cells (10^3–10^6 per mouse, as indicated in each experiment) were transferred i.v. into naive mice and 1 day later, mice were challenged with PR8-OVA at a dose that was lethal for the mice that had not received T cells. *A*, Mouse survival is shown representative of three experiments. *B*, H&E staining of lung sections at day 7 postinfection of mice that had received 10^6, 10^3, or no OT-I T cells (n = 3–4 mice per group). *C*, Analysis of T cell numbers in different tissues is shown. Each data point represents an individual animal. p values were determined by unpaired t test. *D*, Effector function of gated CD45.1$^+$ CD8$^+$ T cells from the lungs is shown. Representative plots of n = 5 mice per group at day 7 postinfection (day 8 after transfer of 5×10^3 or 10^6 T cells) are indicated. FMO controls (*right*) were used to set gates on the *left*. Proportion of total CD8 T cells in each quadrant is indicated. *E*, Survival curve after transfer of 10^3 or 10^4 naive OT-I T cells into WT or 4-1BBL$^{-/-}$ mice, 24 h before PR8-OVA infection (n = 5–6 mice per group and representative of four experiments at 10^3 T cells per mouse). A total of 10^3 OT-I WT or Bim$^{-/-}$ naive OT-I T cells

with no detectable 4-1BB expression on CD8 T cells in the lungs of X31-infected mice (Fig. 6*A*), which had fully cleared the virus by this time point (Fig. 1*B*). At day 8 postinfection, 4-1BB was expressed on the $NP_{366-374}$-specific CD8 T cells in lungs, MLN, and spleen of PR8-infected but little or no 4-1BB was expressed on T cells from X31-infected mice (Fig. 6*B*). Increasing the X31 dose 10-fold to 50 HAU still did not result in significant expression of 4-1BB at day 8 in lung (Fig. 6*A*), likely because the mice are still able to handle this higher dose of virus and clear it rapidly, as evidenced by mouse survival and recovery from weight loss (data not shown). Thus, 4-1BB expression is sustained on the lung CD8 T cells of PR8-infected as compared with X31-infected mice, correlating with delayed viral clearance.

4-1BBL expression is difficult to detect as its expression is transient and it can be released from cells by metalloproteinase cleavage (43). Thus, we were unable to detect specific 4-1BBL protein expression in influenza infected WT as compared with 4-1BBL-deficient mice by flow cytometry or immunofluorescent staining of frozen sections. However, using semiquantitative PCR, we found that there is a low level of 4-1BBL message in lungs of uninfected mice (Fig. 6*C*) and infection with Influenza A/PR8 leads to increased 4-1BBL message. In contrast, Influenza X31 failed to induce significant levels of 4-1BBL message over the level observed in the lungs of uninfected mice. This expression of 4-1BBL in uninfected lung may reflect exposure of the lung to environmental insults. As this experiment measures 4-1BBL mRNA in whole lung normalized to β-actin, the failure to detect increased 4-1BBL mRNA in the lung upon X31 infection may reflect APC migration out of the lung, influx of non-4-1BBL expressing cells, as well as death of infected cells, so that only a more substantial and sustained induction of 4-1BBL by influenza A/PR8 is detected. Thus, PR8 infection leads to higher and more sustained expression of 4-1BBL than X31 infection of mice.

The level of CD8 T cell response critically determines protection from lethal influenza virus infection

Increased CD8 T cell numbers in the lungs of 4-1BBL-sufficient compared with 4-1BBL-deficient mice correlates with improved viral clearance, improved lung function and mouse survival. These observations suggest that an increased CD8 T cell response during a severe viral infection is protective. To test this protective role, we took advantage of an adoptive transfer model using OT-I TCR transgenic T cells specific for OVA together with a recombinant PR8 virus carrying the $OVA_{257-264}$ T cell epitope in its neuraminidase stalk (30). Transfer of 10^3 OT-I T cells to WT mice before challenge with Influenza A/PR8-OVA (PR8-OVA) protected the mice against an otherwise lethal dose of the virus. In contrast, transfer of 10^4 or more T cells was not as protective (Fig. 7*A*). Analysis of cellular infiltration by H&E staining of lung sections showed that there was similar inflammation at sites proximal to the large airways (data not shown), but more extensive inflammation was observed distal to the large airways when 10^6 as compared with 10^3 OT-I T cells were transferred before viral infection (Fig. 7B). Consistent with these results, a higher number of T cells was recovered from the lungs of mice that had received 10^6 as compared with 5×10^3 OT-I T cells (Fig. 7*C*) and these cells were if anything more functional than cells from mice that received less cells, as measured by CD107a and IFN-γ staining following ex vivo peptide stimulation (Fig. 7*D*). Thus, transfer of an optimal number of naive T cells protects against an otherwise lethal dose of influenza, but transfer of too many T cells results in disseminated inflammation and results in mouse death likely due to CD8 T cell-mediated pathology.

In contrast to the effects on WT mice, transfer of 10^3 OT-I T cells to 4-1BBL-deficient mice resulted in only partial protection of mice (Fig. 7*E* left, 20% survival compared with 100% mortality in untreated mice, data not shown). Increasing the number of transferred cells to 10^4 resulted in an even worse outcome (100% fatality by day 8, Fig. 7*E*, *right*). Thus, 4-1BBL in the host is required for adoptively transferred TCR transgenic CD8 T cells as well as endogenous CD8 T cells to protect the host from influenza-induced disease.

Signaling by 4-1BB enhances CD8 T cell survival through the NF-κB mediated up-regulation of Bcl-x_L and Bfl-1 (44, 45) as well as through TNFR-associated factor 1-mediated down-modulation of Bim (46, 47). Thus, we reasoned that the lack of protection of OT-I T cells in 4-1BBL-deficient mice may reflect that 4-1BBL is required in the lung to prolong T cell survival sufficiently to mediate viral clearance. Therefore, we predicted that Bim-deficient T cells would be more protective in 4-1BBL-deficient mice. Indeed, transfer of Bim-deficient OT-I T cells to the 4-1BBL-deficient animals, although unable to fully rescue the 4-1BBL-deficient mice, resulted in a delay in mortality of 1–3 days in three different experiments (Fig. 7*E*, *lower panel*, and data not shown). This experiment suggests that T cell persistence in the lung plays a role in protection from influenza virus, although other factors may also contribute.

The level of 4-1BBL in the lungs is critical in determining disease outcome following severe influenza infection

We attempted to correct the defect in survival of 4-1BBL-deficient mice by systemic administration of agonistic anti-4-1BB Abs. However, this resulted in accelerated disease (data not shown), likely because anti-4-1BB can target many cell types, which leads to excessive cytokine production and immunopathology (48). We therefore attempted a more controlled delivery of 4-1BBL to the lungs, using a recombinant adenovirus expressing 4-1BBL (4-1BBL-AdV). Initial experiments showed that high doses (10^7–10^9 PFU) of 4-1BBL-AdV delivered after influenza infection actually exacerbated disease (data not shown), likely due to increased inflammation. Therefore we titrated the dose of recombinant adenovirus down to 10^5 PFU. The i.n. delivery of 4-1BBL-AdV 2 days before infection with PR8 prolonged the survival of the 4-1BBL-deficient mice by 2 days (Fig. 8*A*). Similarly, 4-1BBL-deficient mice receiving 4-1BBL-AdV 2 days after influenza infection were found to have a slight survival advantage over the group of mice that received the control adenovirus (Control-AdV). In contrast to the results obtained with 4-1BBL-deficient mice, the same dose of 4-1BBL-AdV delivered to WT mice led to increased mortality (Fig. 8*A*).

We next attempted to determine the protective level of 4-1BBL by monitoring 4-1BBL expression following i.n. delivery of the AdV. When 10^9 PFU of 4-1BBL AdV were delivered i.n. to mice, we could readily detect 4-1BBL expression on $CD45^-$ as well as $CD45^+$ lung cells at both day 3 (Fig. 8*B*) and day 5 (data not shown) postinfection by i.n. adenovirus. However, we detected no

(*right*) were injected i.v. into WT or 4-$1BBL^{-/-}$ mice 24 h before infection with PR8-OVA. In three experiments with $n = 3$–4 mice per group, $Bim^{-/-}$ OT-I T cells consistently conferred a 1- to 3-day delay in death of 4-$1BBL^{-/-}$ mice compared with mice that were given WT OT-I T cells. Results with $Bim^{-/-}$ or WT OT-I T cells transferred into WT mice showed variable results, with $Bim^{-/-}$ OT-I T cells showing impaired outcome compared with WT OT-I in some experiments (data not shown). Survival curves were analyzed by the log-rank test, with values for $p < 0.05$ indicated.

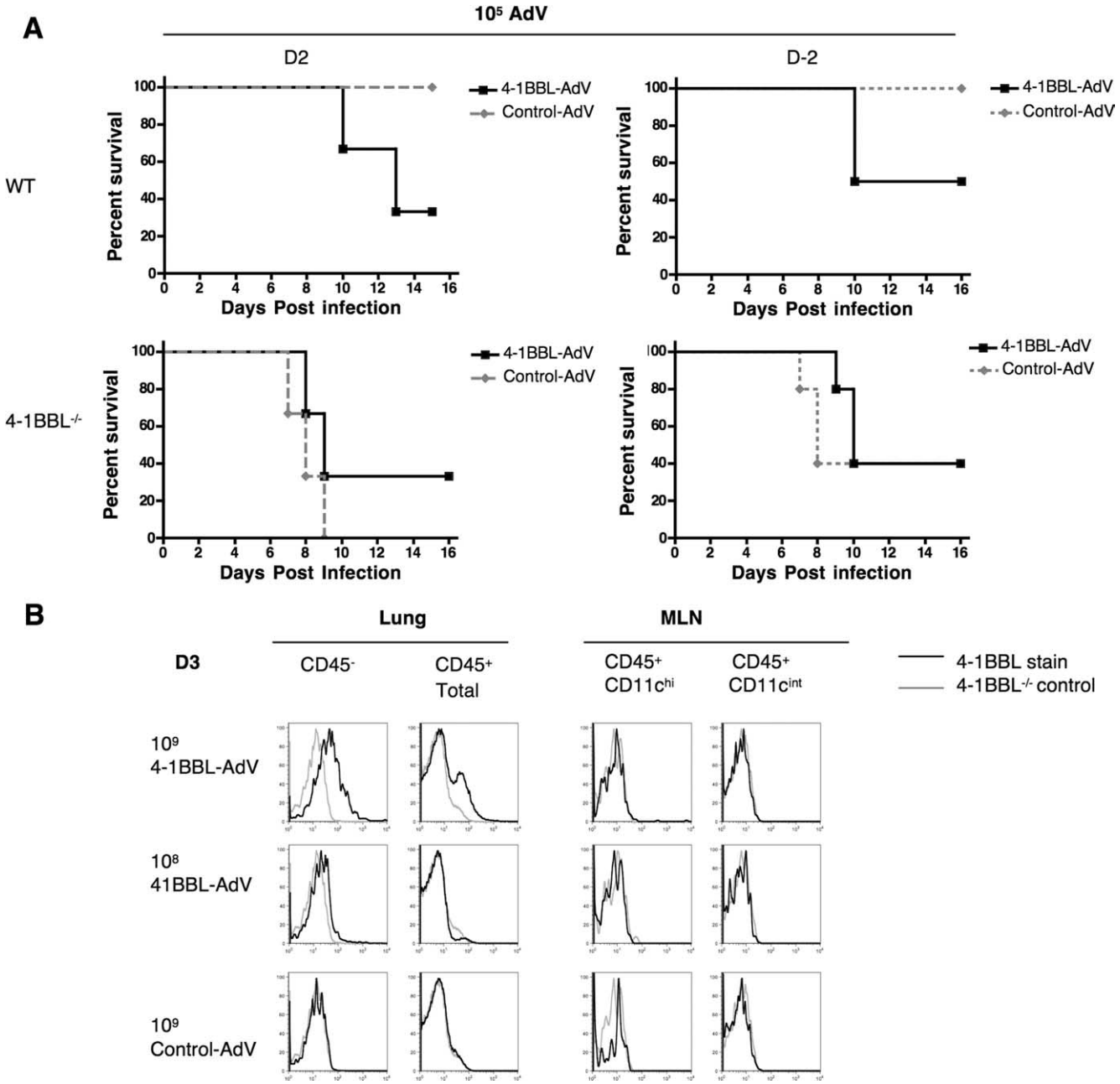

FIGURE 8. The i.n. delivery of 4-1BBL-AdV exacerbates disease in WT mice and at low dose provides limited protection in 4-1BBL-deficient mice. *A*, Survival curve data (n = 2–5 mice per group) following i.n. delivery of 10^5 PFU of adenovirus 2 days after or before infection with PR8 are plotted. *B*, Expression of 4-1BBL in lungs, draining lymph node, and spleen (data not shown) was analyzed on day 3 following i.n. delivery of 10^7–10^9 PFU of adenovirus expressing 4-1BBL or 10^9 PFU of control adenovirus. Similar results were obtained at day 5 (data not shown). Annexin V-positive cells were excluded from the analysis. The histograms are representatives of n = 2–3 mice per group per time point.

expression of 4-1BBL in draining lymph nodes or spleen of the mice (Fig. 8*B*), consistent with the adenovirus inducing localized expression in the lungs. The ability to detect AdV-delivered 4-1BBL fell off rapidly as the dose of adenovirus was lowered below 10^8 PFU per mouse. Thus, the mildly protective dose of 10^5 PFU for 4-1BBL-deficient mice, is below the limit of detection by flow cytometry, likely explaining our inability to detect endogenous 4-1BBL in the lungs of influenza infected WT animals, even though it is required for protective immunity. By titrating the level of 4-1BBL that is detectable by flow cytometry using adenovirus and demonstrating that the protective dose of 4-1BBL delivered exogenously is 3 to 4 logs lower than that which we can detect in the lung by flow cytometry after ectopic expression using an adenovirus vector, we establish that the protective level of endogenous 4-1BBL for survival against influenza is very low.

Discussion

The immune system must balance the level of T cell response to control infection while avoiding immune pathology. In this study we showed that during a mild respiratory influenza infection in which virus is rapidly cleared, 4-1BBL is dispensable for CD8 T cell priming and mouse survival, and 4-1BB is only expressed transiently on the lung T cells. In contrast, with a more virulent influenza infection, resulting in sustained virus levels in the lungs, 4-1BB expression is sustained on CD8 T cells in the lungs and

4-1BBL is critically required for protection. A striking aspect of these results is how a subtle difference in viral clearance in the 4-1BBL-deficient mice (0.7 log or 5-fold) can result in the difference between life and death of the animal. These studies suggest that inducible costimulatory molecules such as 4-1BB may have been acquired by the immune system to handle progressively more virulent infections, so as to maintain the effector CD8 T cell responses when required, but avoid excessive immunity when the virus is readily cleared.

The requirement for 4-1BBL in the protective immune response to influenza virus depends on viral virulence rather than epitope studied, as both the subdominant and the dominant epitopes showed similar sensitivity to the requirement for 4-1BBL in the severe as compared with the mild influenza infection models. In contrast, agonistic anti-4-1BB Abs were shown to diversify the response to influenza virus in mice, by increasing the response to subdominant epitopes (21). This difference may reflect that a supraphysiological 4-1BB signal delivered systemically can enhance the subdominant responses above a threshold, under conditions in which the dominant responses may already be close to maximal.

The major effect of 4-1BBL in this study was on recovery of CD8 effector T cells in the lungs, with no detectable effect on the number of other 4-1BB-expressing subsets including NK, NKT, plasmacytoid dendritic cells, $CD11c^{high}$, $CD11c^{int}$ (monocytes and dendritic cells), CD4, or $\gamma\delta$ T cells. Although transfer of naive Ag-specific TCR transgenic CD8 T cells was protective against an otherwise lethal dose of influenza virus in WT mice, the same was not true in 4-1BBL-deficient mice. Thus 4-1BBL in the host is required for maximal CD8 T cell recovery in the lungs of influenza-infected mice and mouse survival.

4-1BBL could contribute to increased cell recovery in the lungs by increasing T cell expansion, trafficking to the lungs, retention or localization in the lungs or survival of the effector cells in the lungs. Previous studies have shown a major effect of 4-1BB signaling on T cells in vivo is on survival rather than cell division and that anti-4-1BB treatment shows predominant effects directly on the T cells rather than host cells (47). The 4-1BB signaling increases T cell survival (24, 49) through the NF-κB dependent up-regulation of Bcl-x_L and Bfl-1 (44, 45) and through the TNFR-associated factor 1- and ERK-dependent down-regulation of the proapoptotic molecule Bim (46, 47). Consistent with these findings, transfer of Bim-deficient OT-I T cells prolonged the survival of the 4-1BBL-deficient mice by 1–3 days compared with transfer of OT-I WT T cells. Because Bim down-modulation only represents one aspect of 4-1BBL-induced survival signaling (47) and because other mechanisms besides Bim lead to T cell contraction in acute infection (50), it is perhaps not surprising that Bim deficiency does not fully correct the defect. Notwithstanding, given that we detect 4-1BB directly on the T cells in the lung under conditions where 4-1BBL is required for maximal T cell recovery and because genetic ablation of Bim enhances mouse survival, the simplest interpretation of our data is that 4-1BBL increases CD8 T cell numbers in the lung at least in part by directly signaling to the T cells to enhance their survival long enough clear the virus. However, additional scenarios are also possible.

Expression of VLA-1 and LFA-1 on activated and memory CD8 T cells during influenza infection are important in their accumulation in the lung, contributing to increased adhesion and survival (29, 40). Therefore, it was possible that 4-1BBL binding to 4-1BB influences CD8 T cell retention in the lung by influencing T cell activation leading to increased adhesion. However, we detected no significant difference in CD44, 4-1BB, VLA-1, or LFA-1 levels on Ag-specific T cells from WT or 4-1BBL-deficient mice. Thus 4-1BBL largely influences the number of cells in the lung rather than the level of activation per cell.

The engagement of 4-1BBL on APC or myeloid precursors by 4-1BB-Fc fusion proteins has been shown to enhance cytokine production by human macrophages and to increase monocyte differentiation and survival (51–53). Conversely, recent reports have suggested that signaling through 4-1BBL on murine myeloid precursors limits their differentiation to monocytes and dendritic cells (45, 54). Regardless of whether 4-1BBL is a positive or negative regulator of monocyte numbers, we did not detect significant effects of 4-1BBL deficiency on monocyte/macrophage or dendritic cell numbers in the lungs of infected mice (see Supplemental Fig. S2).[5] However, because monocytes and dendritic cells express both receptor and ligand (51, 55), it is formally possible that 4-1BBL-deficiency leads to loss of signals in DC or monocytes that in turn limit their participation in the antiviral response.

Although endogenous 4-1BBL clearly influences the number of T cells in the lungs of mice during severe influenza disease, surprisingly, transfer of extra Ag-specific naive T cells before infection did not correct the defect in 4-1BBL-deficient mice. In WT mice, we found that prior transfer of 1000 Ag-specific naive T cells protected against an otherwise lethal dose of influenza, but increasing the number of Ag-specific CD8 T cells resulted in disseminated inflammation and a worse outcome. TNF production is an early event in CD8 T cell activation (56) and TNF is an important contributor to inflammation during respiratory influenza infection (39). Because TNF production is acquired by naive T cells before the acquisition of CTL killing function, we speculate that increasing the number of naive T cells before infection with influenza in the 4-1BBL-deficient mice may contribute to pathology before effects of 4-1BBL on T cell survival and viral clearance can be realized.

Anti-4-1BB Abs can enhance CD8 T cell responses to viruses (20, 21, 57), and a single dose of anti-4-1BB Ab can completely correct the CD8 memory defect in 4-1BBL-deficient mice in a nonlethal influenza model (20). However, attempts to correct the defect in response to severe influenza in 4-1BBL-deficient mice by systemic administration of anti-4-1BB agonistic Abs were unsuccessful, and resulted in accelerated disease (data not shown). This result is likely because anti-4-1BB can target many cell types and lead to high level cytokine production and immunpathology (48). Using a recombinant adenovirus expressing 4-1BBL to deliver 4-1BBL locally, we showed that a low level of 4-1BBL in the lungs can marginally prolong survival of 4-1BBL-deficient mice following severe influenza infection. The amount of adenovirus that induced a therapeutic effect, 10^5 PFU, was 3 to 4 logs lower than the amount needed to readily detect 4-1BBL protein expression in the lungs. Furthermore, in the present study, adding too much 4-1BBL-expressing recombinant adenovirus to 4-1BBL-deficient mice or providing additional 4-1BBL to WT mice during influenza infection actually impaired outcome. These findings clearly show that the level of endogenous 4-1BBL that is protective in severe influenza infection is very low, and below the limit of detection at the protein level, although detected at the mRNA level. Increasing the level of 4-1BBL to a level detectable by flow cytometry using ectopic expression with an adenovirus vector actually exacerbates disease. Together these findings suggest that the optimal level of 4-1BBL on the surface of cells for protective immunity during an inflammatory lung infection is very low and must be tightly controlled by the immune system to prevent immune pathology. Similar results have been made with respect to CD70 expression, which shows limited protein expression during influenza infection (58) and CD70 transgenic overexpression leads to severe immune system abnormalities (59, 60).

In this study, T cells primed in 4-1BBL-deficient mice under mild or severe influenza infection conditions were fully capable of recall expansion upon transfer and challenge in a new host (see Supplemental Fig. S4).[5] Similar results were also obtained in a previous study, using i.p. infection with influenza A/X31 (20). Thus, 4-1BBL is not required for programming of CD8 T cell memory to a mild or a more severe respiratory influenza infection, but rather controls T cell numbers. However, another study, which used influenza A/NT/60/68, gave different results. The A/NT/60/68 virus does not induce weight loss or inflammation in mice (61), yet deficiency of OX40 ligand or 4-1BBL during priming resulted in CD8 T cells that were impaired in response to influenza virus as well as in programming T cells for secondary expansion (61). Although the influenza NP gene in A/PR8 or X31 and A/NT/60/68 are 94% identical, the major CD8 NP epitope is processed much less efficiently in NT/60/68 than in A/PR8 (62). Thus it is possible that inefficient presentation of this epitope combined with the very low infectivity of this virus in mice results in suboptimal induction of other costimulators such as B7-1 and B7-2, which in turn results in greater dependence on TNFR family costimulation. Further support that this virus is particularly costimulation dependent comes from the observation that OX40 is not required for the primary or secondary CD8 T cell response to Influenza A/PR8 (63), but OX40 ligand is required for the primary response of the A/NT/60/68 virus (61).

The effect of other members of the TNFR family in response to influenza virus in the lungs has also been examined. Deficiency of OX40 was found to impair the CD4 but not the CD8 T cell response to influenza virus, resulting in decreased CD4 T cells accumulating in the lungs (63); however, effects on disease outcome were not reported. Treatment of mice with an OX40-Fc blocking agent following i.n. infection with influenza A X31 resulted in more rapid recovery from weight loss in the mice, as well as decreased CD4 and to a lesser extent CD8 T cell proliferation and increased T cell apoptosis. Thus in contrast to 4-1BBL, which is protective, OX40 appears to play a proinflammatory, immunopathologic role in respiratory influenza infection. CD27 is also important in controlling CD4 and CD8 T cell numbers in the lungs of influenza infected mice (61, 64, 65), although the effect of CD27 on disease outcome during respiratory influenza infection has not been reported.

Why is 4-1BB inducible with TCR signaling, rather than constitutively expressed like CD28? The regulation of 4-1BB expression by Ag-receptor signaling allows an additional level of immune control for infections that take longer to clear while avoiding immune pathology. In the present study, when virus was rapidly cleared, 4-1BB expression was transient, whereas with a virus that took longer to clear, 4-1BB expression on lung T cells was sustained. Thus, the accumulation of inducible receptor/ligand pairs by the mammalian immune system offers a degree of control of the immune response that appears to be dictated by the duration of Ag persistence, which in turn affects the duration of Ag receptor signaling and costimulatory receptor expression. In NOD mice, a polymorphism in the 4-1BB gene that correlates with disease progression has been shown to influence T cell responses (66, 67). In humans, there is also evidence for polymorphism in the 4-1BB locus and it is tempting to speculate that if these polymorphisms lead to differential 4-1BB expression, it may result in differential responses to severe respiratory infections across populations (68).

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Disclosures

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References

1. Wong, P., and E. G. Pamer. 2003. CD8 T cell responses to infectious pathogens. *Annu. Rev. Immunol.* 21: 29–70.
2. Thomas, P. G., R. Keating, D. J. Hulse-Post, and P. C. Doherty. 2006. Cell-mediated protection in influenza infection. *Emerg. Infect. Dis.* 12: 48–54.
3. Doherty, P. C., S. J. Turner, R. G. Webby, and P. G. Thomas. 2006. Influenza and the challenge for immunology. *Nat. Immunol.* 7: 449–455.
4. Moskophidis, D., and D. Kioussis. 1998. Contribution of virus-specific $CD8^+$ cytotoxic T cells to virus clearance or pathologic manifestations of influenza virus infection in a T cell receptor transgenic mouse model. *J. Exp. Med.* 188: 223–232.
5. Humphreys, I. R., G. Walzl, L. Edwards, A. Rae, S. Hill, and T. Hussell. 2003. A critical role for OX40 in T cell-mediated immunopathology during lung viral infection. *J. Exp. Med.* 198: 1237–1242.
6. Wissinger, E., J. Sladana, A. Didierlaurent, and T. Hussell. 2008. Manipulation of acute inflammatory lung disease. *Mucosal Immunology* 1: 265–278.
7. Mercado, R., S. Vijh, S. E. Allen, K. Kerksiek, I. M. Pilip, and E. G. Pamer. 2000. Early programming of T cell populations responding to bacterial infection. *J. Immunol.* 165: 6833–6839.
8. Badovinac, V. P., and J. T. Harty. 2006. Programming, demarcating, and manipulating $CD8^+$ T-cell memory. *Immunol. Rev.* 211: 67–80.
9. Schwartz, R. H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* 71: 1065–1068.
10. Mescher, M. F., J. M. Curtsinger, P. Agarwal, K. A. Casey, M. Gerner, C. D. Hammerbeck, F. Popescu, and Z. Xiao. 2006. Signals required for programming effector and memory development by $CD8^+$ T cells. *Immunol. Rev.* 211: 81–92.
11. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu. Rev. Immunol.* 23: 515–548.
12. Sharpe, A. H., E. J. Wherry, R. Ahmed, and G. J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat. Immunol.* 8: 239–245.
13. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat. Rev. Immunol.* 2: 116–126.
14. Watts, T. H. 2005. TNF/TNFR family members in costimulation of T cell responses. *Annu. Rev. Immunol.* 23: 23–68.
15. Watts, T. H., and M. A. DeBenedette. 1999. T cell costimulatory molecules other than CD28. *Curr. Opin. Immunol.* 11: 286–293.
16. Kwon, B. S., and S. M. Weismann. 1989. cDNA sequences of two inducible T-cell genes. *Proc. Natl. Acad. Sci. USA* 86: 1963–1967.
17. Vinay, D. S., and B. S. Kwon. 1998. Role of 4-1BB in immune responses. *Semin. Immunol.* 10: 481–489.
18. Croft, M. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat. Rev. Immunol.* 3: 609–620.
19. Dawicki, W., and T. H. Watts. 2004. Expression and function of 4-1BB during CD4 versus CD8 T cell responses in vivo. *Eur. J. Immunol.* 34: 743–751.
20. Bertram, E. M., W. Dawicki, B. Sedgmen, J. L. Bramson, D. H. Lynch, and T. H. Watts. 2004. A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza in vivo. *J. Immunol.* 172: 981–988.
21. Halstead, E. S., Y. M. Mueller, J. D. Altman, and P. D. Katsikis. 2002. In vivo stimulation of CD137 broadens primary antiviral $CD8^+$ T cell responses. *Nat. Immunol.* 3: 536–541.
22. Melero, I., W. W. Shuford, S. A. Newby, A. Aruffo, J. A. Ledbetter, K. E. Hellstrom, R. S. Mittler, and L. Chen. 1997. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med.* 3: 682–685.
23. Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Siadak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, et al. 1997. 4-1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification *in vivo* of cytotoxic T cell responses. *J. Exp. Med.* 186: 47–55.
24. Takahashi, C., R. S. Mittler, and A. T. Vella. 1999. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. *J. Immunol.* 162: 5037–5040.
25. Bertram, E. M., P. Lau, and T. H. Watts. 2002. Temporal segregation of CD28 versus 4-1BBL-mediated costimulation: 4-1BBL influences T cell numbers late in the primary response and regulates the size of the memory response following influenza infection. *J. Immunol.* 168: 3777–3785.
26. Pulle, G., M. Vidric, and T. H. Watts. 2006. IL-15-dependent induction of 4-1BB promotes Ag-independent CD8 memory T cell survival. *J. Immunol.* 176: 2739–2748.
27. DeBenedette, M. A., T. Wen, M. F. Bachmann, P. S. Ohashi, B. H. Barber, K. L. Stocking, J. J. Peschon, and T. H. Watts. 1999. Analysis of 4-1BB ligand-deficient mice and of mice lacking both 4-1BB ligand and CD28 reveals a role for 4-1BB ligand in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J. Immunol.* 163: 4833–4841.

28. Tan, J. T., J. K. Whitmire, R. Ahmed, T. C. Pearson, and C. P. Larsen. 1999. 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. *J. Immunol.* 163: 4859–4868.
29. Ray, S. J., S. N. Franki, R. H. Pierce, S. Dimitrova, V. Koteliansky, A. G. Sprague, P. C. Doherty, A. R. de Fougerolles, and D. J. Topham. 2004. The collagen binding $\alpha1\beta1$ integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* 20: 167–179.
30. Jenkins, M. R., R. Webby, P. C. Doherty, and S. J. Turner. 2006. Addition of a prominent epitope affects influenza A virus-specific $CD8^+$ T cell immunodominance hierarchies when antigen is limiting. *J. Immunol.* 177: 2917–2925.
31. Cottey, R., C. A. Rowe, and B. S. Bender. 2001. Measurement of tissue culture influenza virus infectious dose. In *Current Protocols in Immunology*, Vol. 4. John Wiley and Sons, p. 9.11.11.
32. Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham. 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA* 91: 8802–8806.
33. Bukczynski, J., T. Wen, K. Ellefsen, J. Gauldie, and T. H. Watts. 2004. Costimulatory ligand 4-1BBL (CD137L) as an efficient adjuvant for human antiviral cytotoxic T cell responses. *Proc. Natl. Acad. Sci. USA* 101: 1291–1296.
34. Salazar, E., and J. H. Knowles. 1964. An analysis of pressure-volume characteristics of the lungs. *J. Appl. Physiol.* 19: 97–104.
35. Ennis, F. A., M. Verbonitz, P. Reichelderfer, and S. Daniel. 1976. Recombination of influenza A virus strains: effect on pathogenicity. *Dev. Biol. Stand.* 33: 220–225.
36. Allan, W., Z. Tabi, A. Cleary, and P. C. Doherty. 1990. Cellular events in the lymph node and lung of mice with influenza: consequences of depleting $CD4^+$ T cells. *J. Immunol.* 144: 3980–3986.
37. Effros, R. B., P. C. Doherty, W. Gerhard, and J. Bennink. 1977. Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. *J. Exp. Med.* 145: 557–568.
38. Bonville, C. A., N. J. Bennett, M. Koehnlein, D. M. Haines, J. A. Ellis, A. M. DelVecchio, H. F. Rosenberg, and J. B. Domachowske. 2006. Respiratory dysfunction and proinflammatory chemokines in the pneumonia virus of mice (PVM) model of viral bronchiolitis. *Virology* 349: 87–95.
39. Hussell, T., A. Pennycook, and P. J. Openshaw. 2001. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. *Eur. J. Immunol.* 31: 2566–2573.
40. Thatte, J., V. Dabak, M. B. Williams, T. J. Braciale, and K. Ley. 2003. LFA-1 is required for retention of effector CD8 T cells in mouse lungs. *Blood* 101: 4916–4922.
41. Pollok, K. E., Y.-J. Kim, Z. Zhou, J. Hurtado, K. K. Kim, R. T. Pickard, and B. S. Kwon. 1993. Inducible T cell antigen 4-1BB: analysis of expression and function. *J. Immunol.* 150: 771–781.
42. Doherty, P. C., and J. P. Christensen. 2000. Accessing complexity: the dynamics of virus-specific T cell responses. *Annu. Rev. Immunol.* 18: 561–592.
43. Salih, H. R., H. M. Schmetzer, C. Burke, G. C. Starling, R. Dunn, R. Pelka-Fleischer, V. Nuessler, and P. A. Kiener. 2001. Soluble CD137 (4-1BB) ligand is released following leukocyte activation and is found in sera of patients with hematological malignancies. *J. Immunol*. 167: 4059–4066.
44. Arch, R. H., and C. B. Thompson. 1998. 4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kB. *Mol. Cell Biol.* 18: 558–565.
45. Lee, H. W., S. J. Park, B. K. Choi, H. H. Kim, K. O. Nam, and B. S. Kwon. 2002. 4-1BB promotes the survival of $CD8^+$ T lymphocytes by increasing expression of Bcl-x_L and Bfl-1. *J. Immunol.* 169: 4882–4888.
46. Wang, C., T. Wen, J. P. Routy, N. F. Bernard, R. P. Sekaly, and T. H. Watts. 2007. 4-1BBL induces TNF receptor-associated factor 1-dependent Bim modulation in human T cells and is a critical component in the costimulation-dependent rescue of functionally impaired HIV-specific CD8 T cells. *J. Immunol.* 179: 8252–8263.
47. Sabbagh, L., G. Pulle, Y. Liu, E. N. Tsitsikov, and T. H. Watts. 2008. ERK-dependent Bim modulation downstream of the 4-1BB-TRAF1 signaling axis is a critical mediator of CD8 T cell survival in vivo. *J. Immunol.* 180: 8093–8101.
48. Niu, L., S. Strahotin, B. Hewes, B. Zhang, Y. Zhang, D. Archer, T. Spencer, D. Dillehay, B. Kwon, L. Chen, A. T. Vella, and R. S. Mittler. 2007. Cytokine-mediated disruption of lymphocyte trafficking, hemopoiesis, and induction of lymphopenia, anemia, and thrombocytopenia in anti-CD137-treated mice. *J. Immunol.* 178: 4194–4213.
49. Hurtado, J. C., Y. J. Kim, and B. S. Kwon. 1997. Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. *J. Immunol.* 158: 2600–2609.
50. Green, D. R. 2008. Fas Bim boom! *Immunity* 28: 141–143.
51. Langstein, J., J. Michel, J. Fritsche, M. Kreutz, R. Andreesen, and H. Schwarz. 1998. CD137 (ILA/4-1BB), a member of the TNF receptor family, induces monocyte activation via bidirectional signaling. *J. Immunol.* 160: 2488–2494.
52. Langstein, J., and H. Schwarz. 1999. Identification of CD137 as a potent monocyte survival factor. *J. Leukocyte Biol.* 65: 829–833.
53. Jiang, D., Y. P. S. Yue, D. Drenkard, and H. Schwarz. 2008. Induction of proliferation and monocytic differentiation of human $CD34^+$ cells by CD137 ligand signaling. *Stem Cells* 26: 2372–2381.
54. Lee, S. W., Y. Park, T. So, B. S. Kwon, H. Cheroutre, R. S. Mittler, and M. Croft. 2008. Identification of regulatory functions for 4-1BB and 4-1BBL in myelopoiesis and the development of dendritic cells. *Nat. Immunol.* 9: 917–926.
55. Futagawa, T., H. Akiba, T. Kodama, K. Takeda, Y. Hosoda, H. Yagita, and K. Okumura. 2002. Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *Int. Immunol.* 14: 275–286.
56. Brehm, M. A., K. A. Daniels, and R. M. Welsh. 2005. Rapid production of TNF-α following TCR engagement of naive CD8 T cells. *J. Immunol.* 175: 5043–5049.
57. Zhang, B., C. H. Maris, J. Foell, J. Whitmire, L. Niu, J. Song, B. S. Kwon, A. T. Vella, R. Ahmed, J. Jacob, and R. S. Mittler. 2007. Immune suppression or enhancement by CD137 T cell costimulation during acute viral infection is time dependent. *J. Clin. Invest.* 117: 3029–3041.
58. Arens, R., K. Tesselaar, P. A. Baars, G. M. van Schijndel, J. Hendriks, S. T. Pals, P. Krimpenfort, J. Borst, M. H. van Oers, and R. A. van Lier. 2001. Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFNγ-mediated B cell depletion. *Immunity* 15: 801–812.
59. Tesselaar, K., R. Arens, G. M. van Schijndel, P. A. Baars, M. A. van der Valk, J. Borst, M. H. van Oers, and R. A. van Lier. 2003. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. *Nat. Immunol.* 4: 49–54.
60. Tesselaar, K., Y. Xiao, R. Arens, G. M. van Schijndel, D. H. Schuurhuis, R. E. Mebius, J. Borst, and R. A. van Lier. 2003. Expression of the murine CD27 ligand CD70 in vitro and in vivo. *J. Immunol.* 170: 33–40.
61. Hendriks, J., Y. Xiao, J. W. Rossen, K. F. van der Sluijs, K. Sugamura, N. Ishii, and J. Borst. 2005. 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 expansion. *J. Immunol.* 175: 1665–1676.
62. Potter, P., S. Tourdot, T. Blanchard, G. L. Smith, and K. G. Gould. 2001. Differential processing and presentation of the H-$2D^b$-restricted epitope from two different strains of influenza virus nucleoprotein. *J. Gen. Virol.* 82: 1069–1074.
63. Kopf, M., C. Ruedl, N. Schmitz, A. Gallimore, K. Lefrang, B. Ecabert, B. Odermatt, and M. F. Bachmann. 1999. OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL Responses after virus infection. *Immunity* 11: 699–708.
64. Hendriks, J., L. A. Gravestein, K. Tesselaar, R. A. W. van Lier, T. N. M. Schumacher, and J. Borst. 2000. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat. Immunol.* 1: 433–440.
65. Hendriks, J., Y. Xiao, and J. Borst. 2003. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J. Exp. Med.* 198: 1369–1380.
66. Lyons, P. A., W. W. Hancock, P. Denny, C. J. Lord, N. J. Hill, N. Armitage, T. Siegmund, J. A. Todd, M. S. Phillips, J. F. Hess, S. L. Chen, et al. 2000. The NOD *Idd9* genetic interval influences the pathogenicity of insulitis and contains molecular variants of *Cd30*, *Tnfr2*, and *Cd137*. *Immunity* 13: 107–115.
67. Cannons, J. L., G. Chamberlain, J. Howson, L. J. Smink, J. A. Todd, L. B. Peterson, L. S. Wicker, and T. H. Watts. 2005. Genetic and functional association of the immune signaling molecule 4-1BB (CD137/TNFRSF9) with type 1 diabetes. *J. Autoimmun.* 25: 13–20.
68. Maier, L. M., D. J. Smyth, A. Vella, F. Payne, J. D. Cooper, R. Pask, C. Lowe, J. Hulme, L. J. Smink, H. Fraser, et al. 2005. Construction and analysis of tag single nucleotide polymorphism maps for six human-mouse orthologous candidate genes in type 1 diabetes. *BMC Genet.* 6: 9.
69. Townsend, A. R., J. Rothbard, F. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44: 959–968.
70. Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, et al. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nat. Med.* 7: 1306–1312.
71. Vitiello, A., L. Yuan, R. W. Chesnut, J. Sidney, S. Southwood, P. Farness, M. R. Jackson, P. A. Peterson, and A. Sette. 1996. Immunodominance analysis of CTL responses to influenza PR8 virus reveals two new dominant and subdominant Kb-restricted epitopes. *J. Immunol.* 157: 5555–5562.
72. Belz, G. T., W. Xie, and P. C. Doherty. 2001. Diversity of epitope and cytokine profiles for primary and secondary influenza a virus-specific CD8+ T cell responses. *J. Immunol.* 166: 4627–4633.