Analysis of 4-1BB Ligand (4-1BBL)-Deficient Mice and of Mice Lacking Both 4-1BBL and CD28 Reveals a Role for 4-1BBL in Skin Allograft Rejection and in the Cytotoxic T Cell Response to Influenza Virus

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The activation of resting T cells requires both a signal through the TCR and an additional costimulatory signal. It is now well established that the binding of the CD28 receptor on T cells to its ligands B7-1 and B7-2 on APC provides the primary costimulatory signal for high level IL-2 production and survival of primary T cells (1). However, examination of immune responses in mice lacking CD28 revealed that not all responses are CD28 dependent (2–5). In particular, the immune response to some infectious agents, including lymphocytic choriomeningitis virus (LCMV) and the parasite Heligmosomoides polygyrus (4) are unimpaired in CD28−/− mice. 4-1BBL binds to 4-1BB (CD137) on activated CD4 and CD8 T cells and in conjunction with strong signals through the TCR provides a CD28-independent costimulatory signal leading to high level IL-2 production by primary resting T cells. Here we report the immunological characterization of mice lacking 4-1BB and of mice lacking both 4-1BB and CD28. 4-1BB−/− mice mount neutralizing IgM and IgG responses to vesicular stomatitis virus that are indistinguishable from those of wild-type mice. 4-1BB−/− mice show unimpaired CTL responses to lymphocytic choriomeningitis virus (LCMV) and exhibit normal skin allograft rejection but have a weaker CTL response to influenza virus than wild-type mice. 4-1BB−/−CD28−/− mice retain the CTL response to LCMV, respond poorly to influenza virus, and exhibit a delay in skin allograft rejection. In agreement with these in vivo results, allogeneic CTL responses of CD28−/− but not CD28+/− T cells to 4-1BBL-expressing APC are substantially inhibited by soluble 4-1BB receptor as is the in vitro secondary response of CD28−/− T cells to influenza virus peptides. TCR-transgenic CD28−/− LCMV glycoprotein-specific T cells are insensitive to the presence of 4-1BB when a wild-type peptide is used, but the response to a weak agonist peptide is greatly augmented by the presence of 4-1BBL. These results further substantiate the idea that different immune responses vary in their dependence on costimulation and suggest a role for 4-1BBL in augmenting suboptimal CTL responses in vivo. The Journal of Immunology, 1999, 163: 4833–4841.
stimulation of the T cells (17). Costimulation through 4-1BB can augment both Th2 and Th1 cytokine production by T cells (11, 17) and can enhance cytotoxic T cell responses both in vivo and in vitro (18–21). CD8+ T cells proliferate more strongly to anti-CD3 plus anti-4-1BB than do CD4+ T cells (18). In contrast, CD4+ T cells proliferate more vigorously to anti-CD28 than do CD8+ T cells (18, 22). Thus, CD28 and 4-1BB may have complementary roles with respect to both the timing of their effects and their relative effects on CD4 vs CD8 T cell expansion.

In this report, we describe the generation of 4-1BB−/− mice and the characterization of immune responses in these mice as well as in mice doubly deficient for 4-1BB and CD28. We have examined two CD28-independent responses (the CTL response to LCMV (2) and skin allograft rejection (3)) as well as two responses that have been shown to be dependent on CD28 (the IgG response to vesicular stomatitis virus (VSV) (2) and the CTL response to influenza virus (23)). The results demonstrate a role for 4-1BB in the CTL response to influenza virus but show no role for 4-1BB in the CTL response to LCMV or in the neutralizing Ab response to VSV. In the case of skin allograft rejection, the absence of 4-1BB alone has no detectable effect, but doubly deficient CD28−/− 4-1BB−/− mice show a delay in rejection compared with wild-type or single knockout mice. Consistent with the above findings, mice that lack both CD28 and 4-1BB respond to influenza virus (23). The results further substantiate the concept that immune responses to different infectious agents are differentially dependent on costimulation and suggest a role for 4-1BB in augmenting suboptimal CTL responses in vivo.

**Materials and Methods**

**Animals, reagents, and cell lines**

BALB/c and C57BL/6 mice were obtained from Charles River Laboratory (St.-Constant, Quebec, Canada). In addition, C57BL/6 mice were bred in our facility from breeder pairs obtained from Charles River. A/J mice and BALB/c (H-2b) mice (6–8 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME). CD28−/− mice (2) backcrossed onto the C57BL/6 or A/J background (n = 6–10 backcrosses) were provided by Dr. Tak Mak, Amgen Institute, Toronto, Canada, and were bred in our facility. Transgenic mice expressing a TCR specific for the LCMV glycoprotein 33–41 presented in association with H-2b on both a C57BL/6 CD28−/− and C57BL/6 CD28−/− background have been described (6). The LCMV glycoprotein-derived peptide P33 (KAVYNFATM) and the related weak agonist peptide were used. These results further substantiate the concept that immune responses to different infectious agents are differentially dependent on costimulation and suggest a role for 4-1BB in augmenting suboptimal CTL responses in vivo.

**Generation and screening of 4-1BB−/− and 4-1BB−/− CD28−/− mice**

The 4-1BB mutation was moved onto a C57BL/6 background by five successive backcrosses, initiated with (C57BL/6 × 129) F1, 4-1BB−/− mice. The resulting heterozygotes were intercrossed to establish C57BL/6 4-1BB−/− mice. To obtain mice deficient for both the CD28 and 4-1BB genes, homozygous single knockout mice on the C57BL/6 background were bred, F1 littermates were intercrossed, and progeny were genotyped. Genotyping for the 4-1BB mutation was performed by PCR with the following primers: 5′-CAC TGA CCG ACC GTGA GTA ATG-3′ (BBL-1B); 5′-GAC ATA GCG TTG GCT ACC CGT G3-3′ (NEO3′-64); and 5′-AGC CCG GTA TCT CGT AGG AG-3′ (BBL-3). Genotyping for the CD28 mutation was performed by PCR using the following primers: 5′-CTG CTT GTG GTA GAT AGC AAC GA-3′ (CD28ex2); 5′-CCT GAG TCC TGA TCT AGA CT-3′ (CD28in2); and 5′-ATT CGG CAA TGA CAC GAT GG-3′ (HSVTK).

**Analysis of anti-VSV Ab response**

Sera from mice immunized with VSV (Indian strain) were analyzed for neutralizing IgM and IgG Abs as described (27). Briefly, sera was diluted 40-fold in supplemented MEM and heat inactivated for 30 min at 56°C. Twofold serial dilutions were mixed with virus to contain 500 PFU/ml. The mixture was incubated at 37°C for 90 min; then 100 μl were transferred onto VERO cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl DMEM containing 1% methylcellulose, and after 24 h at 37°C the overlay was removed, and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was pre-treated with an equal volume of 0.1 M 2-ME in saline (27).

**Generation of 4-1BB-transfected EL4 cells**

cDNA encoding the full length 4-1BB was obtained by RT-PCR amplification of RNA isolated from the BALB/c B lymphoma K46J, known to express functional 4-1BB (16). Single-stranded cDNA was synthesized from 2 μg total RNA using the First-Strand RNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). PCR was performed with the primers 5′-AAA GGA TCC GGT AAT GGA CCA GCA CAC A-3′ containing the BamHI restriction site and 5′-TGG TCT AGA TTC CCA TTG GTC GTC GG-3′ containing an XhoI restriction. As has been previously reported (12), the extracellular domain of 4-1BB from K46J B lymphomas differs from the previously described sequence of 4-1BB from EL4 cells (14) by a C to A transition at NTD 476, resulting in a change from lysine to glutamine at aa 142. The PCR product was ligated into the pcDNA3 vector (Invitrogen, Carlsbad, CA) using the BamHI/XhoI sites to create the vector pcDNA3.4BL.4. After confirmation of the 4-1BB sequence, EL4 cells were transfected with the vector pcDNA3.4BL.4, selected for neomycin resistance, and sorted for high 4-1BB expression using biotinylated 4-1BB-AP followed by PE-streptavidin (Molecular Probes).

**LCMV-specific T cell responses**

Groups of two mice were infected in the hind foot pad with 300 PFU LCMV-WE strain. After 10 dpi, the primary cell CTL lytic activity was measured against EL4 cells pulsed with the peptide P33 at 0.3 μM in a standard 51Cr release assay as described (2). To evaluate the secondary response to LCMV, mice were infected as above, and on day 20 spleens from mice infected with live virus were harvested and cultured at 4 × 106 cells/ml with 1 × 105 cells/ml Ag-pulsed spleen cells from mice of the same genotype from which the responders were derived. APC were pulsed with P33 at 0.2 μM for 2 h (16) and irradiated (2000 rad) before their addition to the responder cells. Recombinant IL-2 in the form of a culture supernatant from IL-2-transfected X63 cells (28) was added to some cultures at a final concentration of 0.5% (v/v). Stimulated spleen cells were resuspended in 0.5 ml medium per culture well, and serial 3-fold dilutions of effectors were performed (referred to as dilution of standard culture) and tested in a standard 51Cr release assay with EL4 cells pulsed with P33 by PCR, recovered supernatant was counted in a Topcount scintillation counter (Canberra-Packard, Mississauga, Ontario, Canada). Maximum and spontaneous release was determined from wells that contained either 0% SDS.
or medium alone, respectively. Specific lysis was calculated as [(experimental 51Cr release – spontaneous 51Cr release)/(maximum 51Cr release – spontaneous 51Cr release)] × 100. Spontaneous 51Cr release was routinely 5–6% of maximum 51Cr release for each assay.

Analysis of primary responses of TCR transgenic LCMV-glycoprotein-specific T cells in vitro

T cells from either wild-type or CD28−/− mice expressing a transgenic TCR specific for LCMVgp were purified from mouse spleens by lysing RBC with an ammonium chloride solution for 4 min at 37°C. Spleen cell suspensions were resuspended in HBSS containing 2.5% FCS and 50 µM 2-ME. To deplete APC, cell suspensions were incubated with a mixture of Abs, anti-class II (M5K6 or Y3P), anti-B220, anti-HSA (M1/69) and anti-CD11c (N418), each at a final concentration of 15 µg/ml at 4°C for 30 min. A 1:10 dilution of rabbit baby complement (Cedarlane, Hornby, Ontario, Canada) was added, and cultures were further incubated at 37°C for an additional 30 min. Cell suspensions were run over a G-10/nylon wool column to remove adherent cells. Purified T cells were cultured with either EL4 cells or EL4 cells transfected with 4-1BBL, plus 0.2 µM concentration of LCMV-specific peptide A/4Y. CTL activity was assayed 3 days later against A/4Y peptide-pulsed EL4 targets in a conventional 51Cr release assay similar for methods described above.

Influenza virus-specific CTL responses

For analysis of the ability of 4-1BBL to augment the in vitro secondary response to influenza, BALB/c mice were infected i.p. with 200 hemagglutinin units (HAU) of influenza virus strain X-31 (30). X-31 is adapted for replication in chicken eggs and replicates abiotically in mice. Splenocytes were harvested 3 wk postinfection and restimulated in vitro for 7 days with K46J cells pulsed with a 1 µM concentration of the H-2b-restricted peptide nucleoprotein (NP)-147–155 at 37°C for 1 h. K46J lymphoma cells were irradiated (10,000 rad) before their addition to 15-ml cultures. CTL activity was assayed against K46J cells pulsed with NP147–155 and labeled with 200 µCi 51Cr for 90 min. K46J targets (105/well) were added to 2-fold serial dilutions of effectors (100:1–1:1).

For the analysis of the role of 4-1BBL and CD28 in the development of anti-influenza CTL in vivo, C57BL/6, 4-1BBL−/−, CD28−/−, or CD28−/−/4-1BBL−/− (double knockout) mice were infected i.p. with 200 HAU influenza virus strain X-31 as above. Splenocytes were harvested after 3 wk and restimulated in vitro by addition of 2 µM concentrations of the H-2b-restricted peptide NP366–374 to the cultures. On day 7, effector T cells were harvested and assayed for anti-influenza NP-specific CTL activity against 51Cr-labeled EL4 cells pulsed with 50 µM NP366–374 peptide. EL4 target cells (105/well) were added to 3-fold serial dilutions of effectors (100:1–1:1). After 5 h, a 51Cr release assay was performed as described above.

Mixed lymphocyte reactions to generate CTL

Spleen cells from either A/J or wild-type A/J (H-2a) mice were cultured with K46J (H-2d) B lymphoma cells in 25-cm2 tissue culture flasks in an upright position (Corning, Cambridge, MA) for 5 days. Cells were harvested on day 5 for 51Cr release assay. Responders were diluted to give an E:T ratio of 100:1–1:5:1. Allogeneic K46J cells (H-2d) and control BW5.147 cells (H-2d) were labeled with 200 µCi Na125I (100-30 µCi/culture) (Amersham, Oakville, Ontario, Canada) and were used as targets at 1 × 104 cells/well as described above.

Skin tail allografts

Skin tail grafting was performed on anesthetized recipients as described (31). Briefly, a piece of donor tail skin (0.5 cm2 including the epidermis and most of the dermis) was removed with a scalpel and transferred onto the side of the recipient tail from which an equivalent area of skin had been removed. The graft was covered with a clear spray bandage (New-Skin, Medtech Labs, Jackson, WY) and protected with a light loose fitting glass tube. Grafts were visually monitored daily. Grafts were scored as rejected when >80% of the graft area was necrotic.

Results

Generation of 4-1BBL−/− mice by gene targeting

Gene targeting was used to generate 4-1BBL−/− mice as described in Fig. 1, A and B. To verify the absence of 4-1BBL in the mice, lymph node cells from wild-type mice or from 4-1BBL−/− mice were activated for 3 days in Con A and IL-2 followed by 3 h further stimulation with PMA and ionomycin and stained with 4-1BB-Fc. Staining of cells with p75 TNFR-Fc was included as a positive control. Not shown is the absence of any shift using an irrelevant Fc.

Unimpaired anti-VSV neutralizing Ab response in 4-1BBL−/− mice

Previous results have shown that the production of neutralizing IgM against VSV is CD28 independent, whereas the absence of CD28 reduces the titer of neutralizing IgG activity observed at day...
4-1BBL does not appear to be essential for the generation of T-dependent Ab responses or for Ig class switch.

Role of 4-1BBL in the cytotoxic T cell response to LCMV

The immune response to LCMV in mice is characterized by a CD28-independent cytolytic T cell response mediated by CD8 T cells (2). The CD28 independence of this response has been attributed to the high rate of viral replication leading to a prolonged interaction of Ag with the TCR and a reduced requirement for costimulation (6). Alternatively, it is conceivable that prolonged signaling through the TCR might allow time for up-regulation of alternate costimulatory receptors such as 4-1BB. If this were the case, then one might expect a dependence on CD28 costimulation in the absence of 4-1BB, but not in its presence. However, examination of primary responses to LCMV at 8 days after viral infection or examination of the secondary CTL response 20 days after infection revealed no differences between wild-type and 4-1BBL−/− mice or between CD28−/− and 4-1BBL−/− CD28−/− mice (Fig. 4). Furthermore, measurement of viral titers in the spleens of three WT, three 4-1BBL−/−, three CD28−/−, or three doubly deficient mice 4 days postinfection revealed no differences in the ability of these mice to clear the virus (data not shown). Thus, 4-1BBL does not appear to play a role in the primary or the secondary CTL response to LCMV in the presence or absence of CD28.

To explore the potential role of 4-1BBL in augmenting an LCMV-specific CTL response, we conducted additional in vitro experiments using T cells isolated from transgenic mice expressing a TCR specific for the major epitope of LCMV glycoprotein presented by D6 (LCMVgp TCR Tg mice). The cytotoxic response of LCMVgp TCR Tg T cells is CD28 independent when a wild-type peptide, P33, is used whereas when a weak agonist peptide, A4Y, is used, the response becomes CD28 dependent (32). Therefore, we used the weaker A4Y peptide presented by EL4 (H-2b) thymoma cells with and without 4-1BBL to test the potential role of 4-1BBL in augmenting a suboptimal CTL response. Untransfected EL4 cells expressed no detectable 4-1BBL or B-7 family members, whereas 4-1BBL-transfected EL4 cells expressed high levels of 4-1BBL but had also up-regulated B7-1 to low levels (Fig. 5A and data not shown). Therefore, to isolate the effects of 4-1BBL from those of B7-1, we used CD28−/− TCR transgenic T cells for these studies. Fig. 5B shows that CD28−/− LCMVgp-specific T cells respond poorly to A4Y peptide presented by EL4 alone but show a strong primary CTL response to A4Y peptide presented by 4-1BBL-transfected EL4 cells. Furthermore, this response is blocked by inclusion of soluble 4-1BB receptor in the culture (Fig. 5C). Although 4-1BB-AP blocks the development of the CTL effectors, inclusion of 4-1BB-AP during the 4-h 51Cr release assay had no effect on killing, indicating that 4-1BBL acts at the level of development of the CTL effectors rather than in the killing assay per se (data not shown). These results show that 4-1BBL can augment the CTL response to LCMV when a suboptimal signal through the TCR is used. Furthermore, this effect is independent of a signal through the CD28 molecule.

To further explore the effect of 4-1BBL stimulation on the LCMV-specific response, we examined expression of markers of
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Unimpaired primary and secondary responses to LCMV in 4-1BBL−/− and 4-1BBL−/− CD28−/− mice. Groups of two mice were infected in the foot pad with 300 PFU LCMV-WE strain, and 8 days later (A), ○, primary ex vivo CTL lytic activity was measured against EL4 cells pulsed with 0.2 μM peptide P33 in a 51Cr release assay as described in Materials and Methods. □, CTL lytic activity of uninfected mice for each group. Mouse strains are indicated at the top of each panel. Dko. mice deficient in both CD28 and 4-1BBL. This experiment is representative of three similar experiments. B, Secondary responses to LCMV infection were assayed 20 days postinfection by culturing spleen cells from infected mice with irradiated stimulators. Stimulators for each experiment were Ag pulsed (P33 0.2 μM) spleen cells from mice of the same genotype as that used for the responders. ●, Cultures received 0.5% (v/v) IL-2 SN; ○, cultures left unsupplemented. −−−− in B, control lysis of targets without peptide. Five days later, CTL activity was assayed as described above. Uninfected mice gave no responses above background (data not shown). The results shown are representative of two similar experiments.

FIGURE 5. Augmentation of a suboptimal primary CTL response to LCMV-related A4Y peptide by 4-1BB ligand. A, Flow cytometric analysis of EL4 and 4-1BBL-transfected EL4 cells (EL4-4BL). Cells were stained with biotinylated 4-1BB-AP or AP control followed by PE-streptavidin. The shaded histogram represents EL4-4BL cells stained with biotinylated AP. Thin lines represent untransfected EL4 cells, and thick lines represent 4-1BBL-transfected EL4 cells both stained with biotinylated 4-1BB-AP. B, T cells were purified from CD28−/− mice expressing a TCR transgene specific for LMCVgp. T cells (106) were cultured with 105 A4Y peptide-pulsed irradiated EL4 or EL4.4BL cells for 3 days, after which time cells were washed and tested for killing of 51Cr-labeled A4Y-pulsed EL4 targets. Results are representative of three similar experiments. C, Blocking of LCMV peptide-specific CTL response by soluble 4-1BB receptor. T cells (5 × 103/well) were cultured with either 1 × 105 EL4 cells or 1 × 104 EL4-4BL stimulator cells as described in B. Where indicated, 10 μg/ml AP control or 4-1BB-AP (to block 4-1BBL-4-1BB interaction) were added at the onset of culture. Killing was measured as described in Materials and Methods with the use of 51Cr-labeled A4Y-pulsed EL4 targets. Results presented are the average of duplicates and are representative of three such experiments.

Role of 4-1BBL in the immune response to influenza virus

Although we found no evidence for a role for 4-1BBL in the in vivo response to VSV or LCMV, the immune response to influenza virus in mice is a relatively weak response compared with that of LCMV and has been previously shown to be CD28 dependent (23). To test the role of 4-1BBL in the immune response to influenza virus, we first examined the effect of 4-1BBL on augmenting an in vitro secondary response to influenza virus. In these experiments, wild-type BALB/c mice were infected with influenza virus strain X-31. Three weeks postinfection, splenocytes were removed and restimulated with the 4-1BBL expressing H-2d B lymphoma, K46J, and NP147–155 peptide, a major epitope in the H-2Kd-restricted response to influenza virus. Inclusion of a soluble form of the 4-1BB receptor, 4-1BB-AP, during this in vitro restimulation partially abrogated the secondary CTL response to influenza virus (Fig. 7). In contrast, inclusion of soluble 4-1BB-AP during the 4-h 51Cr release assay had no effect on the killing (data not shown). Thus, 4-1BBL can play a role in the development of the
secondary response to influenza virus but is not required for killing of the target cells.

Based on the finding that 4-1BBL could augment an in vitro secondary response to influenza virus, we went on to test whether 4-1BBL could influence the development of this CTL response in vivo. In contrast to the LCMV response, which is sufficiently vigorous to detect a primary CTL response after infection, under the conditions used the CTL response to influenza virus X-31 is readily detectable only after further activation of the T cells in vitro. Wild-type or 4-1BBL-deficient mice were infected with 200 HAU of influenza virus strain X-31. Three weeks later, splenocytes were removed and restimulated by addition of peptide for 7 days. T cells were assayed for lysis of syngeneic target cells incubated with peptide. Results of three independent experiments are shown in Fig. 8. 4-1BBL−/− mice show a decreased ability to respond to influenza virus compared with wild-type mice. The magnitude of the defect varied between experiments but appears to correlate with the strength of the response, such that the weaker the overall response in the assay, the greater is the dependence on 4-1BBL. CD28−/− mice or double knockout mice show a larger defect in the CTL response to influenza than 4-1BBL−/− mice (Fig. 8). Thus, both CD28 and 4-1BBL appear to influence the development of the CTL response to influenza virus in vivo, with CD28 playing the larger role.

Role of 4-1BBL in skin allograft rejection

Previous studies have shown that CD28 is not required for skin allograft rejection (3). Therefore, it was conceivable that alternate costimulatory molecules play a role in this process. To test the

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potential role of 4-1BBL in allogeneic CTL responses in vitro, we used K46J (H-2d) B lymphoma cells as stimulators and T cells from A/J CD28−/− or A/J CD28−/− (H-2a) mice as responders. MLC were incubated for 3 days in the presence or absence of soluble 4-1BB receptor (4-1BB-AP) and T cells were analyzed for killing of labeled K46J (H-2d) vs control BW5.147 (H-2k) targets in a 4-h 51Cr release assay (Fig. 9). Although the response of CD28+/− T cells to allogeneic B lymphomas was only moderately sensitive to blocking of 4-1BB-4-1BBL interaction, the response of the CD28−/− T cells to the allogeneic B lymphomas was completely blocked by inclusion of soluble 4-1BB in the cultures. Thus, 4-1BBL and CD28 may play redundant roles in allogeneic CTL responses.

To test the role of 4-1BBL in allo-responses in vivo, wild-type C57BL/6, 4-1BBL−/−, CD28−/−, and 4-1BB−/−CD28−/− mice, all on the C57BL/6 background, were given tail skin allografts with minor or major histocompatibility differences. CD28−/− and 4-1BB−/− mice were indistinguishable from wild-type mice in their ability to reject minor or major histocompatibility locus-disparate skin grafts. In contrast, mice doubly deficient in CD28 and 4-1BBL showed a delay in skin tail graft rejection of up to 14 days compared with single knockout mice for both fully allogeneic and MHC-disparate skin grafts. In contrast, mice doubly deficient in CD28 and 4-1BBL-AP (5 μg/ml) was added at the initiation of the cultures as indicated. Cultures were fed on day 3 by removing 4 ml medium and replacing it with 4 ml fresh medium plus either 5 μg/ml AP or 4-1BB-AP. Cells were harvested on day 5 for 51Cr release assay as described in Materials and Methods. This experiment is representative of three similar experiments set up in triplicate.

Discussion

In this report, we have described the generation and initial characterization of mice lacking 4-1BBL and of mice doubly deficient in 4-1BB and CD28. The results show that 4-1BBL and CD28 both influence the in vivo CTL response to influenza, with CD28 playing the larger role. In contrast, 4-1BB is not required for the CTL response to LCMV. The effect of 4-1BBL deficiency on the CTL response to influenza virus adds to the accumulating data from other studies, showing a role for 4-1BBL in CD8 T cell responses (18–21).

The CD28 independence of the CTL response to LCMV has been attributed to the high level of viral replication together with the high affinity T cell response allowing sustained T cell activation in the absence of a CD28 signal (6, 7). In contrast, influenza strain X-31 replicates abortively in mice; thus, the requirement for both CD28 and 4-1BBL for maximal responses to influenza X31 is consistent with the hypothesis that a high Ag load allows costimulation-independent immune responses. It is conceivable that the CTL response to LCMV is CD28 independent because the duration of signal 1 allows other costimulatory molecules to be up-regulated on the T cell and replace CD28 signaling. Clearly, removal of 4-1BB alone does not influence the response to LCMV. However, other members of the TNFR family, including OX40 and CD27, can also function to sustain T cell activation (34, 35), and thus there may be functional redundancy in these late costimulatory pathways, resulting in a negligible effect when only one member is removed.

We did not observe any effect of 4-1BBL deficiency on two different Ab responses, the neutralizing Ab response to VSV or anti-TNP IgG production after immunization s.c. with TNP-KLH in alum (Fig. 3 and data not shown). Shuford et al. (18) have shown that CD4 T cells proliferate only poorly in response to anti-CD3 plus 4-1BB engagement compared with CD8 T cells, and this may explain the lack of effects of 4-1BBL deficiency on the T-dependent Ab responses examined. However, it is conceivable that different doses of Ag, different adjuvants, or different sites of immunization would reveal more subtle effects of 4-1BBL on Ab responses. In vitro experiments using B lymphomas expressing high levels of 4-1BBL have shown that 4-1BBL can augment a
CD40 ligand-activated isolated B cells from wild-type or role in APC function. We have not specifically examined mono-
this signal on 4-1BBL expression by the monocytes was not es-
induce signaling in human monocytes, although the dependence of
more likely that the lack of 4-1BBL is affecting the CTL response
response of purified TCR transgenic T cells (Fig. 5), we think it
or NK cells. Further studies on the function of NK cells in the absence of 4-1BBL will therefore be of interest.
It is conceivable that the effects of 4-1BBL deficiency on the
response are due to an indirect effect on the APC rather than
to lack of 4-1BB signaling in the T cell. However, given the ob-
servations that 4-1BBL-transfected EL4 cells augment the CTL
response of purified TCR transgenic T cells (Fig. 5), we think it
more likely that the lack of 4-1BB is affecting the CTL response
directly.
A soluble form of 4-1BB receptor, 4-1BB-Fc, has been shown to
induce signaling in human monocytes, although the dependence of
this signal on 4-1BB expression by the monocytes was not estab-
lished (39). Signaling via 4-1BB may therefore also play a
role in APC function. We have not specifically examined mono-
cyte function in 4-1BB−/− mice, but splenic dendritic cells or
CD40 ligand-activated isolated B cells from wild-type or
4-1BB−/− mice did not show differences in activating LCMV-
specific TCR-transgenic T cells (data not shown). Furthermore,
CD40L-activated splenocytes from wild-type or 4-1BB−/− mice
induced a similar MLR (data not shown). Nevertheless, even after
activation, APC express only low levels of 4-1BB; therefore,
further investigation of where and when 4-1BB is expressed dur-
ing immune responses in vivo will be required before this question
can be properly addressed.
The data presented in this report show that CD28 and 4-1BB can contribute both to skin allograft rejection and to the CTL re-
response to influenza virus in vivo. In the case of influenza virus,
4-1BB and CD28 appear to play nonoverlapping roles, because
removal of either alone is detrimental to the CTL response. In con-
trast, loss of both CD28 and 4-1BB was required before an
effect on skin allograft rejection was observed, suggesting a par-
tially overlapping function for these two costimulatory pathways.
Neither CD28 nor 4-1BB are required for the CTL response to
LCMV. Nevertheless, 4-1BB can enhance the primary response of TCR-transgenic T cells to a suboptimal LCMV-related peptide.
Thus, evidence is accumulating that 4-1BB−/− mice are more likely to be at the level of tumor killing; rather, NK cells appear
appear to be at the level of tumor killing; rather, NK cells appear
survival of fully allogeneic skin and cardiac allografts for
contrast, simultaneous blockade of both CD28 and CD40 leads to
est effect on allograft rejection in two quite different models. In
These data are consistent with the results of Shuford et al. (18) who
showed that systemic administration of agonistic anti-4-1BB Abs
had no effect. 4-1BBL deficiency does not appear to influ-
some of these immune responses in vivo will be required before this question

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