The Journal of Immunology

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

Mark A. DeBenedette, Tao Wen, Martin F. Bachmann, Pamela S. Ohashi, Brian H. Barber, Kim L. Stocking, Jacques J. Peschon and Tania H. Watts

J Immunol 1999; 163:4833-4841; ;
http://www.jimmunol.org/content/163/9/4833

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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[1]

Mark A. DeBenedette,* Tao Wen,* Martin F. Bachmann,[2†] Pamela S. Ohashi,*† Brian H. Barber,* Kim L. Stocking,[‡] Jacques J. Peschon,[‡] and Tania H. Watts[3]*

4-1BB ligand (4-1BBL) is a member of the TNF family expressed on activated APC. 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 4-1BBL and of mice lacking both 4-1BBL and CD28. 4-1BBL$^{-/-}$ mice mount neutralizing IgM and IgG responses to vesicular stomatitis virus that are indistinguishable from those of wild-type mice. 4-1BBL$^{-/-}$ 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. 4-1BBL$^{-/-}$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 4-1BBL 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.**

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)[4] (2) and the parasite *Heligmosomoides polygyrus* (4) are unimpaired in CD28$^{-/-}$ mice. In the case of LCMV, this CD28 independence appears to be due to viral replication in the mouse, allowing prolonged signaling through the TCR, thereby bypassing requirement for a CD28 signal (6, 7). Alternatively, it is possible that during a prolonged signal through the TCR, other costimulatory receptors could be up-regulated on the T cell leading to CD28-independent responses.

4-1BB (CD137) is a member of the TNF receptor superfamily expressed on activated CD4 and CD8 T cells (8) as well as on activated NK cells (9). The binding of 4-1BB to its TNF family ligand, 4-1BB ligand (4-1BBL) delivers a costimulatory signal to resting T cells leading to high level IL-2 production independently of CD28 signaling (8, 10–12). 4-1BB mRNA is detectable in resting T cells within 3 h of activation via the TCR, and its surface expression peaks at 40–60 h, declining again by 96 h (8). Abs to 4-1BB have a larger effect on preactivated vs resting T cells and prevent activation-induced death of previously activated T cells (13). When signals through the TCR are high, recombinant 4-1BBL can induce levels of IL-2 production by primary resting T cells that are equivalent to that induced by anti-CD3 plus anti-CD28. However, when signals through the TCR are low, CD28 is much more potent than 4-1BB in costimulation of resting T cells (12). This may be due to the requirement for a strong TCR signal to induce 4-1BB expression.

4-1BBL is expressed on activated APC, including activated B cells, macrophages (14–16), and mature dendritic cells (10). The paucity of 4-1BBL on resting APC as well as the requirement for T cell activation for 4-1BB expression has led to the idea that 4-1BB-4-1BBL plays a role in sustaining T cell responses subsequent to CD28 costimulation (8). This hypothesis is supported by recent experiments with human T cells, which show that signals through 4-1BB can enhance responses to anti-CD3 plus anti CD28 after anti-CD28 responsiveness has been lost due to repetitive

*Department of Immunology, University of Toronto, Toronto, Ontario, Canada; †Department of Medical Biophysics, University of Toronto and the Ontario Cancer Institute, Toronto, Ontario, Canada; and ‡Immunex, Seattle, WA 98101-2936

Received for publication June 1, 1999. Accepted for publication August 24, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a grant from the Medical Research Council of Canada (to T.H.W.).

[2] Current address: Basel Institute for Immunology, Grenzacher Strasse 486, 4005 Basel, Switzerland.

[3] Address correspondence and reprint requests to Dr. Tania H. Watts, Department of Immunology, University of Toronto, Toronto, Ontario, Canada M5S 1A8. E-mail address: tania.watts@utoronto.ca

[4] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; 4-1BBL, 4-1BB ligand; VSV, vesicular stomatitis virus; 4-1BB-AP, 4-1BB-alkaline phosphatase; AP, alkaline phosphatase; LCVMgp TCR Tg, LCMV-glycoprotein-specific TCR transgenic; HAU, hemagglutinin units; KLH, keyhole limpet hemocyanin; TNP, trinitrophenyl; NP, nucleoprotein.

0022-1767/99/$02.00

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 4-1BBL$^{-/-}$ mice and the characterization of immune responses in these mice as well as in mice doubly deficient for 4-1BBL 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 4-1BBL in the CTL response to influenza virus but show no role for 4-1BBL in the CTL response to LCMV or in the neutralizing Ab response to VSV. In the case of skin allograft rejection, the absence of 4-1BBL alone has no detectable effect, but doubly deficient CD28$^{-/-}$4-1BBL$^{-/-}$ mice show a delay in rejection compared with wild-type or single knockout mice. Consistent with the results in 4-1BBL$^{-/-}$ mice, in vitro studies with APC that express high levels of 4-1BBL implicate 4-1BBL in allogeneic T cell responses only in the absence of CD28 costimulation and a role for 4-1BBL in augmenting the CTL response to influenza virus even in the presence of CD28. In the case of LCMV, a role for 4-1BBL in augmenting CTL responses of LCMV glycoprotein-specific TCR receptor transgenic (LCMVgp TCR Tg) T cells in vitro could be demonstrated when a weak agonist peptide but not the wild-type peptide was used. These results further substantiate the concept that immune responses to different infectious agents are differentially dependent on costimulation and suggest a role for 4-1BBL 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/b (H-2^b) 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-2^b 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, A4Y (KAVANFATM) were synthesized at the Amgen Institute (Boulder, CO) as described (6). K46J is a BALB/c B cell lymphoma originally described by Kim et al. (24). 4-1BB:Fc has been described (14). 3T3 cells secreting a fusion protein consisting of the extracellular domain of 4-1BB linked to alkaline phosphatase (AP) was provided by Dr. Byoung Kwon (Indiana University, Indianapolis, IN). 4-1BB-AP was purified on anti-AP-Sepharose as described (25). Anti-CD4 (GK1.5), anti-CD8 (53.6.72), anti-CD25 (PC61.5.3), and anti-CD62L (Mel 14)-secreting hybridomas were obtained from the American Type Culture Collection, and Abs were purified from culture supernatants using protein G-Sepharose (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. PE-CD44 was obtained from PharMingen (San Diego, CA), and PE-streptavidin was from Molecular Probes (Eugene, OR).

Targeting of the 4-1BBL gene by homologous recombination

A genomic clone encoding murine 4-1BBL was isolated from a 129-derived lambda library (Stratagene) and mapped by PCR, restriction, and sequence analyses. A 4-1BBL gene-targeting vector was generated by inserting a PGK-neo cassette into the *Sst*II site at nucleotide 52 of the 4-1BBL gene (14) and a thymidine kinase cassette (TK) at the 5′ end of the vector. 129-derived ES cells were electroporated with the 4-1BBL-targeting vector and selected as described (26). Targeted clones were identified by PCR and Southern blot analyses and injected into C57BL/6 blastocysts. The resulting male chimeras were bred to C57BL/6 females and analyzed for germline transmission of the disrupted 4-1BBL allele.

Generation and screening of 4-1BBL$^{-/-}$ and 4-1BBL$^{-/-}$CD28$^{-/-}$ mice

The 4-1BBL mutation was moved onto a C57BL/6 background by five successive backcrosses, initiated with (C57BL/6 × 129) F_1 4-1BBL$^{+/-}$ mice. The resulting heterozygotes were intercrossed to establish C57BL/6 4-1BBL$^{-/-}$ mice. To obtain mice deficient for both the CD28 and 4-1BBL genes, homozygous single knockout mice on the C57BL/6 background were bred, F_1 littermates were intercrossed, and progeny were genotyped. Genotyping for the 4-1BBL mutation was performed by PCR with the following primers: 5′-CAC TGA CCG ACC GTG GTA ATG-3′ (BBL-1B); 5′-GAC ATA GCG TTG GCT ACC CGT G-3′ (NEO3′-64); and 5′-AGC CCG GTA TCT CTG 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 GTC AGA CT-3′ (CD28in2); and 5′-ATT CGG CAA TGA CAA GAC GTT GG-3′ (HSVTK).

Analysis of anti-VSV Ab response

Sera from mice immunized with VSV (Indiana 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 pretreated with an equal volume of 0.1 M 2-ME in saline (27).

Generation of 4-1BBL-transfected EL4 cells

cDNA encoding the full length 4-1BBL was obtained by RT-PCR amplification of RNA isolated from the BALB/c B lymphoma K46J, known to express functional 4-1BBL (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 *Bam*HI restriction site and 5′-TGC TCT AGA TTC CCA TGG GTT GTC GG-3′ containing an *Xba*I restriction. As has been previously reported (12), the extracellular domain of 4-1BBL from K46J B lymphomas differs from the previously described sequence of 4-1BBL from EL4 cells (14) by a C to A substitution 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 *Bam*HI/*Xba*I sites to create the vector pcDNA3.4BL.4. After confirmation of the 4-1BBL sequence, EL4 cells were transfected with the vector pcDNA3.4BL.4, selected for neomycin resistance, and sorted for high 4-1BBL 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 8 days, primary ex vivo CTL lytic activity was measured against EL4 cells pulsed with the peptide P33 at 0.2 μM in a standard ^{51}Cr release assay as described (2). To evaluate the secondary response to LCMV, mice were infected as above, and on day 20 spleens from infected mice were harvested and cultured at 4×10^6 cells/ml with 1×10^6 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 1 h 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). Restimulated 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 ^{51}Cr release assay with peptide-pulsed EL4 cells. After 5 h, 50 μl of supernatant were harvested and counted in a Topcount scintillation counter (Canberra-Packard, Mississauga, Ontario, Canada). Maximum and spontaneous release was determined from wells that contained either 1% SDS

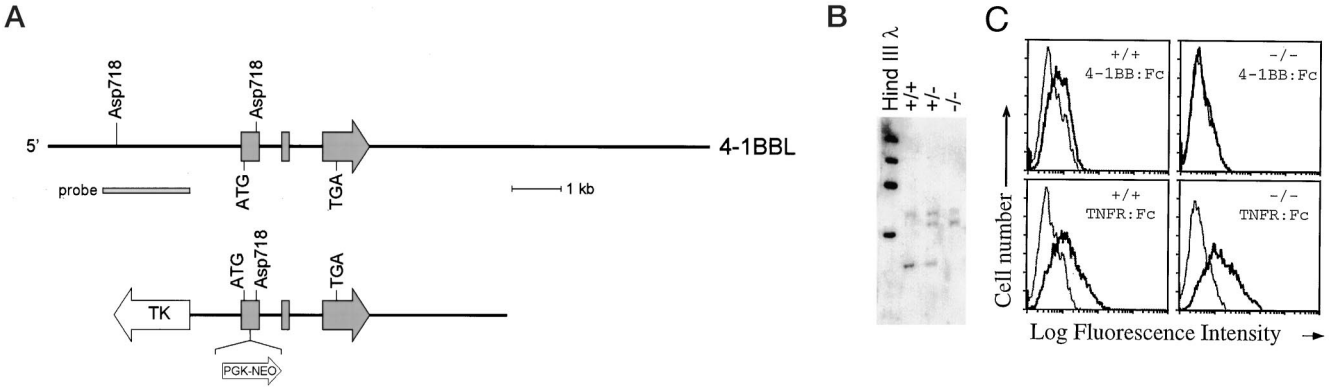

FIGURE 1. Generation and initial characterization of 4-1BBL$^{-/-}$ mice. *A*, Targeting vector used to disrupt the 4-1BBL gene. The initiator methionine (ATG), termination codon (TGA), relevant restriction sites, and the probe used for genomic Southern blot analyses are indicated. Exons are depicted as shaded boxes. *B*, Southern blot of genomic DNAs prepared from wild-type (+/+), 4-1BBL$^{+/-}$ (+/−), and 4-1BBL$^{-/-}$ (−/−) mice; digested with Asp718; and probed with the indicated probe. *C*, Flow cytometry analysis showing the lack of 4-1BB:Fc binding to 4-1BBL-deficient cells. Peripheral lymph node cells from wild-type or 4-1BBL$^{-/-}$ mice were stimulated 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.

or medium alone, respectively. Specific lysis was calculated as [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100. Spontaneous ^{51}Cr release was routinely 5–6% of maximum ^{51}Cr 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 (MKD6 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 baby rabbit 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 A4Y. CTL activity was assayed 3 days later against A4Y peptide-pulsed EL4 targets in a conventional ^{51}Cr 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 strain X-31 produced as described (29). The X-31 strain of influenza virus contains the *NP* gene from A/PR/8/34 virus (30). X-31 is adapted for replication in chicken eggs and replicates abortively 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-2^d-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 ^{51}Cr for 90 min. K46J targets (10^4/well) were added to 2-fold serial dilutions of effectors (200:1–1:1).

For 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 strain X-31 as above. Splenocytes were harvested after 3 wk and restimulated in vitro by addition of 2 μM concentrations of the H-2^b-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 ^{51}Cr-labeled EL4 cells pulsed with 50 μM NP366–374 peptide. EL4 target cells (10^4/well) were added to 3-fold serial dilutions of effectors (100:1–3:1). After 5 h, a ^{51}Cr release assay was performed as described above.

Mixed lymphocyte reactions to generate CTL

Spleen cells from either A/J CD28$^{-/-}$ or wild-type A/J (H-2^a) mice were cultured with K46J (H-2^d) B lymphoma cells in 25-cm^2 tissue culture flasks in an upright position (Corning, Cambridge, MA) for 5 days. Cells were harvested on day 5 for ^{51}Cr release assay. Responders were diluted to give an E:T ratio of 100:1–1.5:1. Allogeneic K46J cells (H-2^d) and control BW5.147 cells (H-2^k) were labeled with 200 μCi $Na_2{}^{51}CrO_4$ (Amersham, Oakville, Ontario, Canada) and were used as targets at 1×10^4 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 cm^2 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 PMA and ionomycin. Activated lymph node cells from wild-type mice expressed a low but reproducible level of 4-1BBL, whereas no 4-1BBL was detectable on activated lymph node cells from 4-1BBL$^{-/-}$ mice (Fig. 1*C*). The 4-1BBL mutation was backcrossed onto the C57BL/6 background, and mice were also crossed with C57BL/6 CD28$^{-/-}$ H-2^b mice to generate mice doubly deficient in 4-1BBL and CD28. Examination of spleen and lymph node organs in the mice showed no obvious differences in size between the wild-type and deficient strains, and yields of cells from spleen and lymph nodes of wild-type and knockout mice were comparable (data not shown). Cell suspensions from lymph node and spleen were compared for expression of T cell and B cell surface markers by flow cytometry (Fig. 2). 4-1BBL$^{-/-}$ mice and 4-1BBL$^{-/-}$ CD28$^{-/-}$ mice were indistinguishable from wild-type mice in terms of CD4$^+$ and CD8$^+$ T cell distribution and had normal populations of splenic B cells and CD8$^+$ dendritic cells (Fig. 2).

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

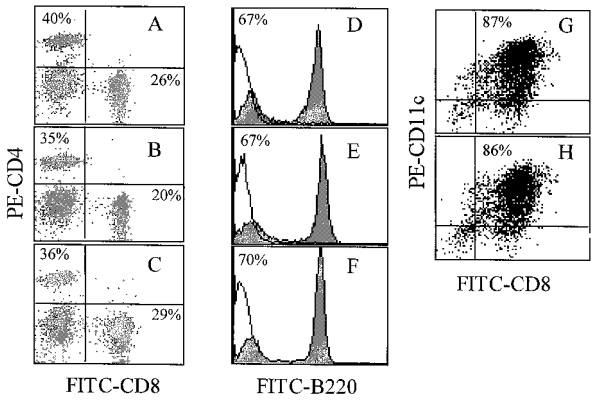

FIGURE 2. Normal distribution of T cells, B cells, and splenic $CD8^+$ dendritic cells in 4-1BBL$^{-/-}$ and double knockout mice. Lymph nodes and spleens were harvested from either wild-type mice (*A*, *D*) 4-1BBL$^{-/-}$ mice (*B*, *E*), or 4-1BBL$^{-/-}$ CD28$^{-/-}$ mice (*C*, *F*). T cells in lymph nodes (*A–C*) were doubly stained with FITC-CD8 and biotin-CD4. Spleen cells were stained with an isotype control Ab (open histograms) or FITC-B220 (dark histograms) (*D–F*). Dendritic cells were purified from wild-type mice (*G*) or 4-1BBL$^{-/-}$ mice (*H*) as described previously (10) by the method of Metlay et al. (41) and double stained with FITC-CD8 and biotin-CD11c. For biotinylated Abs, PE-streptavidin was used as a second step. The percentage of positive cells in each region or quadrant is indicated. Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Samples were monitored for forward vs side scatter and for two fluorescence channels. Propidium iodide staining and forward and side scatter were used to gate on live cells. Data were analyzed and plotted using Becton Dickinson CELLQuest software.

10 after VSV infection. These results suggested a role for CD28 in T cell help leading to Ig class switch (2). Similar experiments with 4-1BBL$^{-/-}$ mice showed no defect in the ability to produce neutralizing IgM or IgG (Fig. 3). In a separate experiment, no difference in IgM or IgG production was observed between CD28$^{-/-}$ and CD28$^{-/-}$ 4-1BBL$^{-/-}$ mice (three mice/group, data not shown). In addition, two wild-type and two 4-1BBL$^{-/-}$ mice were immunized in each flank s.c. with 40 μg trinitrophenyl (TNP)-keyhole limpet hemocyanin (KLH) in alum and analyzed for TNP-specific total IgG, IgG1, and IgG2a after primary and secondary immunization. The responses of the wild-type and 4-1BBL$^{-/-}$ mice were comparable (data not shown). Thus, the presence of 4-1BBL does not appear to be essential for the generation of T-dependent Ab responses or for Ig class switch.

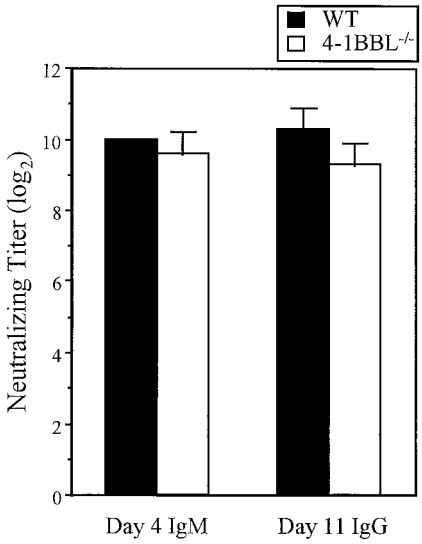

FIGURE 3. Unimpaired neutralizing Ab response to VSV in 4-1BBL$^{-/-}$ mice. Mice were infected i.v. with VSV (Indiana strain, 2×10^6 PFU). Sera from wild-type (WT) or 4-1BBL$^{-/-}$ mice were analyzed on day 4 or day 11 for neutralizing IgM and IgG Abs as described (42). No neutralizing anti-VSV IgG was detectable at day 4 (not shown). Titers represent 2-fold dilution steps of sera starting with 1:40 dilution. Each bar represents the mean value from three mice, and data are expressed as $\log_2$ of the neutralizing titer. Results are from one experiment. A second experiment with two mice per group showed similar results.

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 4-1BBL, 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 D^b (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-2^b) 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. 5*A* 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. 5*B* 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. 5*C*). Although 4-1BB-AP blocks the development of the CTL effectors, inclusion of 4-1BB-AP during the 4-h ^{51}Cr 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

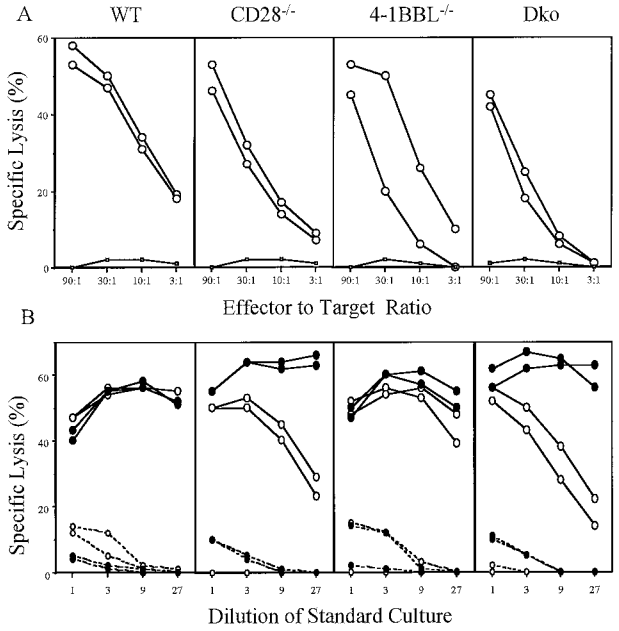

FIGURE 4. Unimpaired primary and secondary responses to LCMV in 4-1BBL$^{-/-}$ and 4-1BBL$^{-/-}$CD28$^{-/-}$ mice. Groups of two mice were infected in the foot pad with 300 PFU LCMV-WE strain, and 8 days later (*A*). ○, primary ex vivo CTL lytic activity was measured against EL4 cells pulsed with 0.2 μM peptide P33 in a ^{51}Cr 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.

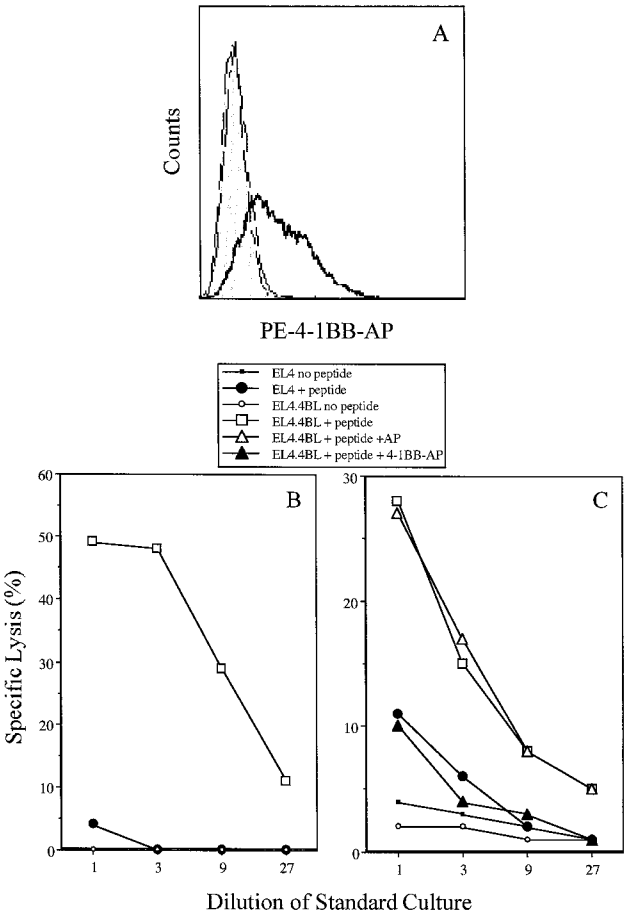

FIGURE 5. Augmentation of a suboptimal primary CTL response to LCMV-related A4Y peptide by 4-1BB ligand. *A*, Flow cytometric analysis of EL4 and 4-1BBL-transfected EL4 cells (EL4-4BL). Cells were stained with biotinylated 4-1BB-AP or AP control followed by PE-streptavidin. The shaded histogram represents 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 (10^6) were cultured with 10^5 A4Y peptide-pulsed irradiated EL4 or EL4.4BL cells for 3 days, after which time cells were washed and tested for killing of ^{51}Cr-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 (5×10^5/well) were cultured with either 1×10^4 EL4 cells or 1×10^4 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 ^{51}Cr-labeled A4Y-pulsed EL4 targets. Results presented are the average of duplicates and are representative of three such experiments.

effector/memory vs naive T cells on TCR-transgenic T cells responding to peptide presented by EL4 or to 4-1BBL-transfected EL4 cells. Fig. 6 shows that by 3 days after incubation with APC plus peptide, TCR-transgenic T cells exhibit a decrease in expression of CD62L (Mel 14), an increase in expression of CD25 and an increase in expression of CD44. The presence of 4-1BBL on the APC markedly enhances these effects compared with incubation with EL4 plus peptide alone. These changes are similar to that reported for LCMVgp transgenic T cells responding to viral peptide presented by spleen cells (33) and are independent of the CD28 costimulatory pathway. Although EL4 cells alone induce some changes in the T cell surface phenotype, in the absence of transfected 4-1BBL, EL4 cells are unable to support expansion of the CD28$^{-/-}$TCR-transgenic T cells or development of CTL effector function (Fig. 5*B*). Thus, 4-1BBL can promote the development of effector/memory CTL in primary cultures of TCR transgenic CD8 T cells.

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-2^d B lymphoma, K46J, and NP147–155 peptide, a major epitope in the H-2K^d-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 ^{51}Cr release assay had no effect on the killing (data not shown). Thus, 4-1BBL can play a role in the development of the

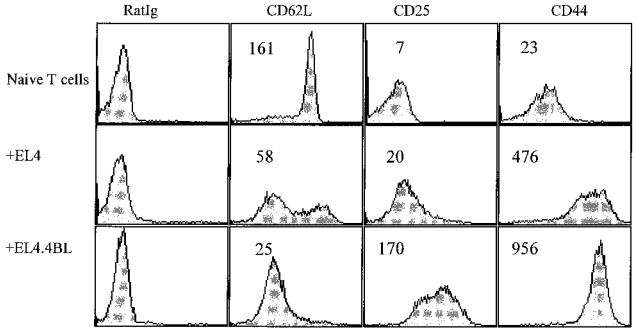

FIGURE 6. Effector memory phenotype of LCMV Tg T cells activated in the presence of 4-1BBL. T cells (5×10^5) were purified from CD28$^{-/-}$ LCMV TCR-transgenic mice and stimulated with 0.2 μM peptide A4Y plus irradiated (12,000 rad) EL4 cells or EL4-4BL cells at 5×10^4 cells/well in 12-well plates. Cell surface staining for the indicated markers was performed on day 3. Live cells were gated on the CD8$^+$ population for all groups. Numbers beside each histogram represent the MFI for the CD8$^+$ cells.

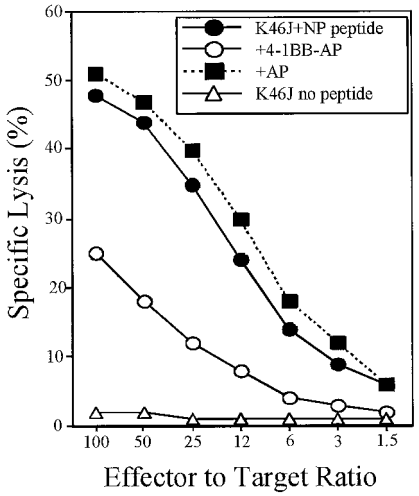

FIGURE 7. 4-1BBL can contribute to the in vitro secondary response to influenza virus. BALB/c mice were infected i.p. with 200 HAU influenza strain X-31. Three weeks later, spleen cells were harvested and restimulated in vitro with irradiated H-2^d K46J B lymphoma cells (10,000 rad) that had been prepulsed with 100 μM concentrations of the H-2K^d-restricted peptide NP147–155. Where indicated, some cultures received either 10 μg/ml AP control or 10 μg/ml 4-1BB-AP. On day 7, effectors were harvested, and anti-influenza NP-specific CTL activity was assayed against ^{51}Cr-labeled K46J cells pulsed with 1 μg NP147–155 peptide. CTL assays were set up as described in *Materials and Methods.* Results are representative of two similar experiments using spleen cells pooled from two mice for each group set up in triplicate.

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

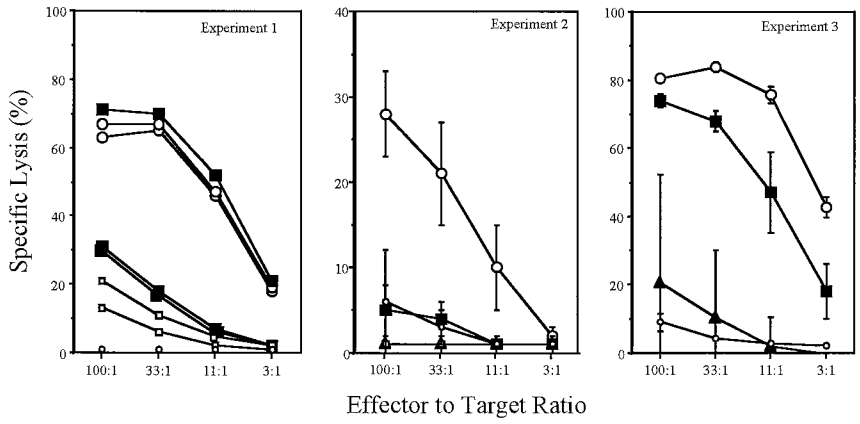

FIGURE 8. 4-1BBL-deficient mice are impaired in their memory CTL response to influenza virus. Groups of two to four mice were infected i.p. with 200 HAU influenza strain X-31. Three weeks later, spleens were harvested and restimulated in vitro with 2 μM concentrations of the H-2^b-restricted peptide NP366–374. On day 7, effectors were harvested, and anti-influenza NP-specific CTL activity was assayed against ^{51}Cr-labeled EL4 cells pulsed with 50 μM NP peptide. CTL assays were set up as described in *Materials and Methods.* Results of three independent experiments are shown. Solid squares, 4-1BBL$^{-/-}$ mice; large open circles, wild-type mice; solid triangles, CD28$^{-/-}$ mice (experiments 2 and 3); small open squares, CD28$^{-/-}$ 4-1BBL$^{-/-}$ mice (experiments 1 and 2); small circles, lytic activity against EL4 cells in the absence of peptide. In experiment 1, individual mice were assayed separately and results of each mouse are shown. In experiments 2 and 3, spleens from 2 (experiment 2) or 4 (experiment 3) infected mice were pooled and restimulated in quadruplicate cultures, and the results are presented as the average of the individual cultures. Error bars indicate the SD between the replicate cultures.

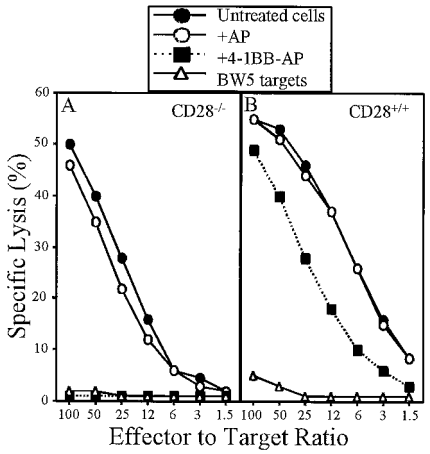

FIGURE 9. Role of 4-1BBL in augmenting an in vitro allogeneic CTL response. Spleen cells (3×10^7) from either A/J CD28$^{-/-}$ mice (*A*) or wild-type A/J mice (H-2^k) (*B*) were cultured in 8 ml complete medium with 3×10^6 irradiated (10,000 rad) K46J B lymphomas (H-2^d). AP or 4-1BB-AP (5 μg/ml) was 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 ^{51}Cr release assay as described in *Materials and Methods*. This experiment is representative of three similar experiments set up in triplicate.

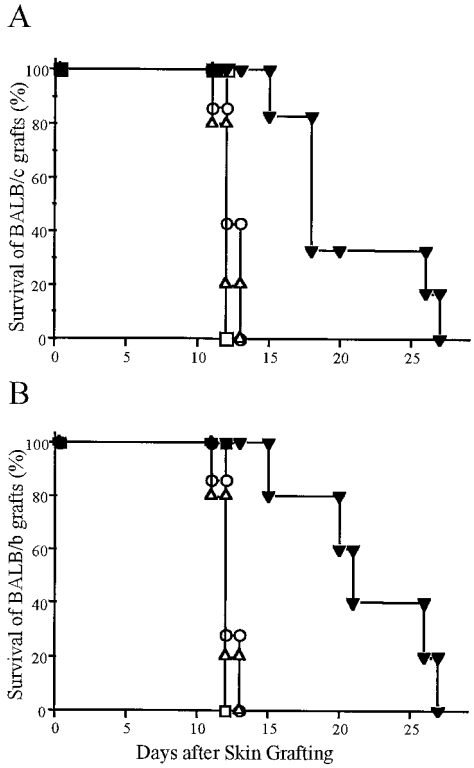

FIGURE 10. Prolonged skin allograft survival in 4-1BBL$^{-/-}$CD28$^{-/-}$ mice. Groups of mice 8–10 wk of age were transplanted with either fully allogeneic tail skin from BALB/c mice (*A*) or minor MHC-incompatible tail skin from BALB/b mice (*B*). □, wild-type C57BL/6 mice ($n = 3$); ○, C57BL/6 CD28$^{-/-}$ mice ($n = 7$); △, C57BL/6 4-1BBL$^{-/-}$ mice ($n = 6$); ▼, double knockout mice ($n = 6$). Results are plotted as Kaplan-Meier graft survival curves.

potential role of 4-1BBL in allogeneic CTL responses in vitro, we used K46J (H-2^d) B lymphoma cells as stimulators and T cells from A/J CD28$^{+/+}$ or A/J CD28$^{-/-}$ (H-2^a) 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-2^d) vs control BW5.147 (H-2^k) targets in a 4-h ^{51}Cr 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 4-1BBL$^{-/-}$CD28$^{-/-}$ mice, all on the C57BL/6 background, were given tail skin allografts with minor or major histocompatibility differences. CD28$^{-/-}$ and 4-1BBL$^{-/-}$ 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 minor histocompatibility locus-disparate grafts (Fig. 10). Thus, lack of both CD28 and 4-1BBL, but lack of neither alone, causes a modest delay in skin graft rejection, suggesting a redundancy in their function in this system.

Discussion

In this report, we have described the generation and initial characterization of mice lacking 4-1BBL and of mice doubly deficient in 4-1BBL 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, 4-1BBL 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 upregulated on the T cell and replace CD28 signaling. Clearly, removal of 4-1BBL 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

Th2 response (11). In other studies, anti-4-1BB has been shown to augment the production of Th1 cytokines (17). However, in our study no differences in IgG1 or IgG2a levels were noted in response to TNP-KLH immunization of 4-1BBL-deficient vs wild-type controls. Thus, 4-1BBL deficiency does not appear to influence the Th phenotype of this response.

CD28 has been shown to be dispensable for allograft rejection (3). In the present studies, we find that lack of both 4-1BBL and CD28 resulted in a delay of up to 14 days in minor and major histocompatibility locus-disparate tail skin allograft rejection, whereas the absence of CD28 or 4-1BBL alone had no effect. These data are consistent with the results of Shuford et al. (18) who showed that systemic administration of agonistic anti-4-1BB Abs results in an acceleration of skin or cardiac allograft rejection by ~3 days. Thus, 4-1BB-4-1BBL interaction appears to have a modest effect on allograft rejection in two quite different models. In contrast, simultaneous blockade of both CD28 and CD40 leads to survival of fully allogeneic skin and cardiac allografts for >50 days (36). CD40 signaling induces up-regulation of a number of costimulatory and accessory molecules including IL-12 (37), 4-1BBL (10), CD27L (38), and OX40L (35), likely explaining the more profound effects of CD40/CD28 blockade compared with the effect of CD28 plus 4-1BBL deficiency.

In addition to being expressed on activated CD4 and CD8 T cells, 4-1BB is also expressed on activated NK cells. Augmentation of tumor rejection by agonistic anti-4-1BB Abs is dependent on $CD4^+$ T cells, $CD8^+$ T cells, and NK cells (9, 19). The effect of NK cells in anti-4-1BB-mediated tumor elimination does not appear to be at the level of tumor killing; rather, NK cells appear to play a regulatory role (9). Thus, the effect of 4-1BBL-deficiency on graft rejection could likewise be due to effects on CD4 T cells, CD8 T cells, and/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 CTL 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 observations 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 4-1BBL 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 4-1BBL expression by the monocytes was not established (39). Signaling via 4-1BBL may therefore also play a role in APC function. We have not specifically examined monocyte function in $4\text{-}1BBL^{-/-}$ mice, but splenic dendritic cells or CD40 ligand-activated isolated B cells from wild-type or $4\text{-}1BBL^{-/-}$ mice did not show differences in activating LCMV-specific TCR-transgenic T cells (data not shown). Furthermore, CD40L-activated splenocytes from wild-type or $4\text{-}1BBL^{-/-}$ mice induced a similar MLR (data not shown). Nevertheless, even after activation, APC express only low levels of 4-1BBL; therefore, further investigation of where and when 4-1BBL is expressed during immune responses in vivo will be required before this question can be properly addressed.

The data presented in this report show that CD28 and 4-1BBL can contribute both to skin allograft rejection and to the CTL response to influenza virus in vivo. In the case of influenza virus, 4-1BBL and CD28 appear to play nonoverlapping roles, because removal of either alone is detrimental to the CTL response. In contrast, loss of both CD28 and 4-1BBL was required before an effect on skin allograft rejection was observed, suggesting a partially overlapping function for these two costimulatory pathways. Neither CD28 nor 4-1BBL are required for the CTL response to LCMV. Nevertheless, 4-1BBL can enhance the primary response of TCR-transgenic T cells to a suboptimal LCMV-related peptide. Thus, evidence is accumulating that 4-1BB-4-1BBL interaction is an important costimulatory pathway for up-regulating suboptimal CD8 T cell responses independently of the CD28 molecule (40).

Acknowledgments

We thank Tak Mak for provision of $CD28^{-/-}$ mice, Arsen Zakarian for help with VSV neutralization and LCMV clearance assays, and Ed Bertram and Jennifer Cannons for critical review of the manuscript.

References

1. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol. 14:233.*
2. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science 261:609.*
3. Kawai, K., A. Shahinian, T. W. Mak, and P. S. Ohashi. 1996. Skin allograft rejection in CD28-deficient mice. *Transplantation 61:352.*
4. Gause, W. C., S. J. Chen, R. J. Greenwald, M. J. Halvorson, P. Lu, X. D. Zhou, S. C. Morris, K. P. Lee, C. H. June, F. D. Finkelman, J. F. Urban, and R. Abe. 1997. CD28 dependence of T cell differentiation to IL-4 production varies with the particular type 2 immune response. *J. Immunol. 158:4082.*
5. Wen, T., K. Kono, A. Shahinian, R. Kiessling, T. W. Mak, and G. Klein. 1997. CD28 is not required for rejection of unmanipulated syngeneic and autologous tumors. *Eur. J. Immunol. 27:1988.*
6. Kundig, T. M., A. Shahinian, K. Kawai, H. W. Mittrucker, E. Sebzda, M. F. Bachmann, T. W. Mak, and P. S. Ohashi. 1996. Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity 5:41.*
7. Bachmann, M. F., R. M. Zinkernagel, and A. Oxenius. 1998. Immune responses in the absence of costimulation: viruses know the trick. *J. Immunol. 161:5791.*
8. Vinay, D. S., and B. S. Kwon. 1998. Role of 4-1BB in immune responses. *Semin. Immunol. 10:481.*
9. Melero, I., J. V. Johnston, W. W. Shufford, R. S. Mittler, and L. Chen. 1998. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell. Immunol. 190:167.*
10. DeBenedette, M. A., A. Shahinian, T. W. Mak, and T. H. Watts. 1997. Costimulation of $CD28^-$ T lymphocytes by 4-1BB ligand. *J. Immunol. 158:551.*
11. Chu, N. R., M. A. DeBenedette, B. J. Stiernholm, B. H. Barber, and T. H. Watts. 1997. Role of IL-12 and 4-1BB ligand in cytokine production by $CD28^+$ and $CD28^-$ T cells. *J. Immunol. 158:3081.*
12. Saoulli, K., S. Y. Lee, J. L. Cannons, W. C. Yeh, A. Santana, M. D. Goldstein, N. Bangia, M. A. DeBenedette, T. W. Mak, Y. Choi, and T. H. Watts. 1998. CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. *J. Exp. Med. 187:1849.*
13. 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.*
14. Goodwin, R. G., W. S. Din, T. Davis-Smith, D. M. Anderson, S. D. Gimpel, T. A. Sato, C. R. Maliszewski, C. I. Brannan, N. G. Copeland, N. A. Jenkins, T. Farrah, R. J. Armitage, W. C. Fanslow, and C. A. Smith. 1993. Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. *Eur. J. Immunol. 1993:2631.*
15. Pollok, K. E., Y.-J. Kim, J. Hurtado, Z. Zhou, K. K. Kim, and B. S. Kwon. 1994. 4-1BB T cell antigen binds to mature B cells and macrophages, and costimulates anti-μ-primed splenic B cells. *Eur. J. Immunol. 24:367.*
16. DeBenedette, M. A., N. R. Chu, K. E. Pollok, J. Hurtado, W. F. Wade, B. S. Kwon, and T. H. Watts. 1995. Role of 4-1BB ligand in costimulation of T lymphocyte growth and its upregulation on M12 B lymphomas by cAMP. *J. Exp. Med. 181:985.*
17. Kim, Y. J., S. H. Kim, P. Mantel, and B. S. Kwon. 1998. Human 4-1BB regulates CD28 co-stimulation to promote Th1 cell responses. *Eur. J. Immunol. 28:881.*
18. 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, T. C. Pearson, J. A. Ledbetter, A. Aruffo, and R. S. Mittler. 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.*
19. Melero, I., W. W. Shuford, S. A. Newby, A. Aruffo, J. A. Ledbetter, K. E. Hellstr[umlaut]om, R. S. Mittler, and L. Chen. 1997. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med. 3:682.*
20. Melero, I., N. Bach, K. E. Hellstrom, A. Aruffo, R. S. Mittler, and L. Chen. 1998. Amplification of tumor immunity by gene transfer of the co-stimulatory 4-1BB ligand: synergy with the CD28 co-stimulatory pathway. *Eur. J. Immunol. 28: 1116.*
21. Guinn, B. A., M. A. DeBenedette, T. H. Watts, and N. L. Berinstein. 1999. 4-1BBL co-operates with B7-1 and B7-2 in converting a B cell lymphoma cell line into a long lasting anti-tumor vaccine. *J. Immunol. 162:5003.*
22. Abe, R., P. Vandenberghe, N. Craighead, D. S. Smoot, K. P. Lee, and C. H. June. 1995. Distinct signal transduction in mouse $CD4^+$ and $CD8^+$ splenic T cells after CD28 receptor ligation. *J. Immunol. 154:985.*

23. Liu, Y., R. H. Wenger, M. Zhao, and P. J. Nielsen. 1997. Distinct costimulatory molecules are required for the induction of effector and memory cytotoxic T lymphocytes. *J. Exp. Med. 185:251.*
24. Kim, K. J., C. Kanellopoulos-Langevin, R. M. Merwin, D. H. Sachs, and R. A. Asofsky. 1979. Establishment and characterization of BALB/c lymphoma lines with B cell properties. *J. Immunol. 122:549.*
25. 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.*
26. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer, and B. L. Davidson. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med. 180:1955.*
27. Oxenius, A., K. A. Campbell, C. R. Maliszewski, T. Kishimoto, H. Kikutani, H. Hengartner, R. M. Zinkernagel, and M. F. Bachmann. 1996. CD40-CD40 ligand interactions are critical in T-B cooperation but not for other anti-viral $CD4^+$ T cell functions. *J. Exp. Med. 183:2209.*
28. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5 using modified cDNA expression vectors. *Eur. J. Immunol. 18:97.*
29. Uger, R. A., and B. H. Barber. 1998. Creating CTL targets with epitope-linked β_2-microglobulin constructs. *J. Immunol. 160:1598.*
30. Baez, M., P. Palese, and E. D. Kilbourne. 1980. Gene composition of high-yielding influenza vaccine strains obtained by recombination. *J. Infect. Dis. 141: 362.*
31. Wen, T., L. Zhang, S. K. P. Kung, T. J. Molina, R. G. Miller, and T. W. Mak. 1995. Allo-skin graft rejection, tumor rejection and natural killer activity in mice lacking $p56^{lck}$. *Eur. J. Immunol. 25:3155.*
32. Bachmann, M. F., K. McKall-Faienza, R. Schmits, D. Bouchard, J. Beach, D. E. Speiser, T. W. Mak, and P. S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity 7:549.*
33. Oehen, S., and K. Brduscha-Riem. 1998. Differentiation of naive CTL to effector and memory CTL: Correlation of effector function with phenotype and cell division. *J. Immunol. 161:5338.*
34. Lens, S. M. A., K. Tesselaar, M. H. J. van Oers, and R. A. W. van Lier. 1998. Control of lymphocyte function through CD27-CD70 interaction. *Semin. Immunol. 10:491.*
35. Weinberg, A. D., A. T. Vella, and M. Croft. 1998. OX-40: life beyond the effector T cell stage. *Semin. Immunol. 10:471.*
36. Larsen, C. P., E. T. Elwood, D. Z. Alexander, S. C. Ritchie, R. Hendrix, C. Tucker-Burden, H. R. Cho, A. Aruffo, D. Hollenbaugh, P. S. Linsley, K. J. Winn, and T. C. Pearson. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature 381:434.*
37. Kato, T., R. Hakamada, H. Yamane, and H. Nariuchi. 1996. Induction of IL-12 p40 messenger RNA expression and IL-12 production of macrophages via CD40-CD40 ligand interaction. *J. Immunol. 156:3932.*
38. Hartwig, U. F., L. Karlsson, P. A. Peterson, and S. R. Webb. 1997. CD40 and IL-4 regulate murine CD27L expression. *J. Immunol. 159:6000.*
39. 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.*
40. Watts, T. H., and M. A. DeBenedette. 1999. T cell costimulatory molecules other than CD28. *Curr. Opin. Immunol. 11:286.*
41. Metlay, J. P., M. D. Witmer-Pack, R. Agger, M. T. Crowley, D. Lawless, and R. M. Steinman. 1990. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med. 171: 1753.*
42. Leist, T. P., S. P. Cobbold, H. Waldmann, M. Aguet, and R. M. Zinkernagel. 1987. Functional analysis of T lymphocyte subsets in antiviral host defense. *J. Immunol. 138:2278.*