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4-1BB (CD137) signals depend upon CD28 signals in alloimmune responses

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Abbreviations: AICD, activation-induced cell death; CFSE, 5,6-carboxylfluorescein diacetate succinimidyl ester; MLR, mixed leukocyte reaction; MST, mean survival time; TCR, T-cell receptor; WT, wild-type

Abstract

Our previous study has demonstrated that there is a significant delay of Balb/c cardiac allograft rejection in the C57BL/6 4-1BB-deficient knockout recipient. In this study, we examined the effect of combined blockade of the 4-1BB and CD28 costimulatory pathways on cardiac allograft rejection in the C57BL/6 → Balb/c model. A long-term cardiac allograft survival was induced in CD28/4-1BB-deficient mice (> 100 days survival in 3 of 4 mice), which was comparable with CD28-deficient mice (> 100 days survival in 2 of 5 mice; *P* < 0.2026). There was no long-term cardiac allograft survival in either wild-type (WT) or 4-1BB-deficient mice, even though 4-1BB-deficient recipients showed a significant delay of cardiac allograft rejection than WT mice. An *in vitro* mixed leukocyte reaction (MLR) assay showed that 4-1BB-deficient and WT mouse T cells had a similar responsiveness to allostimulation, whereas CD28- and CD28/4-1BB-deficient mouse T cells had a defective responsiveness to allostimulation. Furthermore, 4-1BB-deficient mice showed a similar CTL but an elevated Ab response against alloantigens as compared to WT mice, and the alloimmune responses of 4-1BB-deficient mice were abrogated in the CD28-deficient background. Overall, these results indicate that the CD28 costimulatory pathway plays a major role in the alloimmune response and that 4-1BB signals are dependent upon CD28 signals.

Keywords: antibody formation; antigens; CD28; CD137 antigen; graft rejection; mice, knockout

Introduction

Because T cells are the primary mediators of allograft rejection (Hall, 1991; Rosenberg and Singer, 1992), much effort has been directed at designing therapeutics that specifically block the initial activation of T cells in allograft recipients. T cell activation is dependent upon signals generated by the T-cell receptor (TCR) when it recognizes a peptide presented by a MHC molecule. However, the TCR signal fails to fully activate T cells in the absence of a second, costimulaory signal (Schwartz, 1990). A number of receptor/ligand pairs expressed on the surface of T cells and APCs are capable of providing the necessary costimulatory signal.

The 4-1BB/4-1BB ligand (4-1BBL) and CD28/CD80/CD86 pathways are two well characterized costimulatory pathways. Both pathways are critical in the activation and maturation of T cells, and thus have been explored as therapeutic targets in a number of transplant models. Blockade of the CD28 costimulatory pathway has been shown to prevent acute allograft rejection and to induce donor-specific tolerance in several murine transplant models (Turka *et al*., 1992; Pearson *et al*., 1994; Sayegh *et al*., 1995). Treatment with CTLA4-Ig, a recombinant fusion protein containing the extracellular domain of CTLA-4 and a Fc portion of human IgG_1, can inhibit proliferation of alloreactive T cells and induce anergy in those cells that do proliferate despite CD28 blockade (Judge *et al*., 1996). Importantly, activation-induced cell death (AICD) is required for CTLA4-Ig-medaited T-cell tolerance. Agents that increase AICD, such as rapamycin, promote tolerance by

blockade of the CD28/CD40 costimulatory pathways (Li *et al*., 1999). Other observations also support AICD as a prerequisite for the generation of peripheral tolerance to allografts by costimulatory blockade (Dai *et al*., 1998; Wells *et al*., 1999). Recently, a high-affinity variant of CTLA4-Ig has been developed and has shown significant prolongation of renal allograft survival in a pre-clinical primate model (Larsen *et al*., 2005) and human patients (Vincenti *et al*., 2005).

4-1BB (CD137) is a costimulatory member of the TNF receptor (TNFR) family expressed on activated T cells, some dendritic cells (DCs) and activated NK cells (Vinay and Kwon, 1998; Kwon *et al*., 2000; 2002a; 2003). 4-1BBL is expressed on activated APCs, such as B cells, macrophages and DCs (Goodwin *et al*., 1993; Pollok *et al*., 1994; DeBenedette *et al*., 1997; Futagawa *et al*., 2002; Wilcox *et al*., 2002a). Interactions of 4-1BB/4-1BBL were suggested to be involved in alloimmune responses, since both 4-1BB and 4-1BBL transcripts were expressed in rejecting mouse cardiac allografts (Tan *et al*., 2000). Indeed, blockade of the 4-1BB costimulatory pathway was shown to be effective in prevention of $CD8^+$ T-cell-mediated small bowl rejection (Wang *et al*., 2003). We also demonstrated that there was a significant delay in cardiac allograft rejection in 4-1BB-deficient mice (Cho *et al*., 2004). Moreover, anti-4-1BBL mAb induced a long-term allograft survival in 2 out of 5 allograft recipients. It seems that, even though anti-4-1BBL mAb could not block proliferation of T cells initially, it was effective in decreasing the number of alloreactive T cells at the later stage of alloimmune responses (Cho *et al*., 2004). In the chronic graft-versus-host disease (cGVHD) setting, stimulation of 4-1BB was highly effective in preventing and curing cGVHD by inducing AICD of pathogenic donor $CD4^+$ T cells (Kim *et al*., 2005).

Some evidence suggests that 4-1BB might play a role in the immune response later than CD28, and possibly functions to perpetuate the immune response (Kim *et al*., 1998; Rothstein *et al*., 2003). Based on this and other facts that resistance of some $CD8^+$ T cells to treatment with CTLA4-Ig is the main reason for rejection of allografts in several models (Newell *et al*., 1999; Trambley *et al*., 1999) and that 4-1BB is a key costimulatory molecule for $CD8^+$ T cells, we hypothesized that there could be a synergistic effect of combined blockade of the CD28/4-1BB costimulatory pathways on prolongation of cardiac allograft survival.

Materials and Methods

Mice

Breeding pairs of wild-type (WT) Balb/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from the Jackson Laboratory (Bar Habor, ME). CD28-deficient (Lucas *et al*., 1995) and 4-1BB-deficient (Kwon *et al*., 2002b) mice were backcrossed to Balb/c mice for more than 10 generations, and CD28/4-1BB-deficient mice were generated by crossing CD28-deficient with 4-1BB-deficient mice (Vinay *et al*., 2003). All mice were bred in the University of Ulsan Animal Facility. Mice were maintained in a dedicated, specific pathogen-free facility in microisolator cages. We performed all animal experiments following the "Principles of Laboratory Animal Care" (NIH publication No. 86-23).

Heterotopic heart transplantation

Vascularized donor heart grafts from C57BL/6 mice were transplanted into WT, CD28-deficient, 4-1BB-deficient, and CD28/4-1BB-deficient Balb/c mice, essentially as previously described (Corry *et al*., 1973). In brief, donor hearts were harvested and placed in cold PBS until transplanted. The recipient mice were anesthetized, and the aorta and pulmonary artery of the donor hearts were sutured to the recipient aorta and inferior vena cava, respectively, end-to-side using 10-0 suture silk. Graft survival was monitored by daily abdominal palpation. Rejection was considered complete when cardiac contraction had completely ceased. This was confirmed by direct visualization of grafted hearts.

Immunohistochemical analysis

Grafted C57BL/6 hearts were harvested 7 days after transplant. Immunohistochemistry was done as described previously (Kim *et al*., 2006). In brief, the tissues were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrence, CA) and snap-frozen in liquid nitrogen. Sections (8-μm thick) were air-dried, fixed with acetone, and stained sequentially with anti-CD3 mAb, biotyinylated rabbit anti-rat IgG, streptavidin-horseradish peroxidase (HRP), and a substrate for HRP (BD Biosciences Pharmingen, San Diego, CA).

In vitro mixed leukocyte reaction (MLR)

To prepare APC-enriched populations, C57BL/6 splenocytes were incubated in 10-cm plates at 37°C for 2 h to remove non-adherent lymphocytes. The adherent cells were further incubated overnight at 37°C. These APCs were irradiated (30 Gy) before used to stimulate T cells of Balb/c origin. Balb/c T cells were prepared from spleen and lymph node cells using nylon wool column (Wako Chemical, Tokyo, Japan) after red blood cell lysis on Tris-buffered ammonium chloride for 3 min. For an *in*

vitro MLR, responder Balb/c T cells (1×10^5/well) and stimulator C57BL/6 APCs (5×10^5/well) were added in triplicate into 95-well plates in a final volume of 200 μl. The cultures were incubated at 37°C in a humidified air containing 5% CO_2 for 3 d. Proliferation on day 3 was assessed by pulsing with 1 μCi [^{3}H]-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) added during the last 12 h.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from MLR cultures were harvested and the concentrations of IFN-γ and IL-2 were determined according to the manufacturer's protocol (Pierce Endogen, Rockford, IL).

Measurement of allospecific IgG

Allospecific IgG was measured as described previously (Sakurai *et al*., 2000). In brief, serum was harvested from the tail vein at days 0, 7, 14, 21 and 28 after transplantation. EL4 cells (H-2^b) were incubated with each serum on ice. After 20 min, the cells were washed and then incubated with FITC-conjugated anti-mouse IgG (BD Pharmigen Biosciences). After washing two times, the fluorescence intensities were monitored using a FACS and data were analyzed using Cellquest software (BD Pharmingen Biosciences).

In vivo CTL assay

An *in vivo* CTL was modified as described previously (Coles *et al*., 2002). In brief, C57BL/6 mice were irradiated with 8 Gy and received 2×10^7 of WT, CD28-, 4-1BB-, or CD28/4-1BB-deficient splenocytes. After 5 d, target cells were prepared from naïve C57BL/6 and Balb/c splenocyte suspensions by lysing erythrocytes using Tris-buffered ammonium chloride for 3 min. After washing, C57BL/6 cells were labeled with 2.5 μM of 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (CFSE^{high}) and used as target cells, and Balb/c cells were labeled with 0.25 μM of CFSE (CFSE^{low}) and used as control target cells. For reference cells (syngeneic cells), Balb/c splenocytes were labeled with a high or a low concentration of CFSE. For i.v. injection, an equal number of cells from each population was mixed together, such that each mouse received a total of 20×10^6 cells in 150 μl of PBS. Cells were injected into the immunized WT, CD28-deficient, 4-1BB-deficient, and CD28/4-1BB-deficient mice. Four hours later, splenocytes were harvested and analyzed by FACS, and each population was detected by their differential CFSE fluorescence intensities (Kim *et al*., 2006). To calculate allospecific lysis, the following formula was used: ratio = (percentage CFSE^{low}/percentage CFSE^{high}); percentage allospecific lysis = [1-(ratio syngeneic/ratio allogeneic) × 100].

Statistical analysis

Kaplan-Meier survival graphs were constructed and log-rank comparisons of experimental groups were used to calculate *P* values. Significant differences between experimental groups in MLR and ELISA were analyzed using Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

To investigate the effect of 4-1BB/CD28 costimulatory blockade on cardiac allograft survival, we transplanted fully MHC-mismatched C57BL/6 (H-2^b) cardiac allografts into WT, CD28-deficient, 4-1BB-deficient, or CD28/4-1BB-deficient Balb/c (H-2^d) recipients. As shown in the Balb/c→C57BL/6 model (Cho *et al*., 2004), the 4-1BB defect was associated with a significant delay in cardiac allograft rejection (Figure 1); the mean survival time (MST) were 8 and 20 d in WT and 4-1BB-deficient mice, respectively ($n = 7$; $P < 0.0017$). In CD28-deficient mice, the MST was 45 d ($n = 5$; $P < 0.002$) and 2 out of 5 mice had a long-term cardiac allograft survival (> 100 d). There was a long-term allograft survival in 3 out of 4 mice in CD28/4-1BB-deficient mice ($n = 4$; $P = 0.0045$). Statistical analysis revealed no significant difference in allograft survival between CD28-deficient and CD28/4-1BB-deficient mice, indicating that combined blockade of CD28/4-1BB might have no synergistic effect on cardiac allograft rejection. Immunohistological analysis at day 7 after transplan-

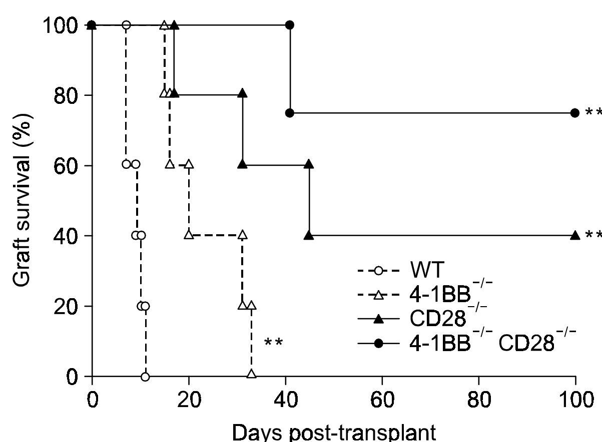


Figure 1. Survival of cardiac allogaft survival. Vascularized C57BL/6 cardiac grafts were transplanted into WT, CD28-, 4-1BB-, and CD28/4-1BB-deficient Balb/c mice. ***P* < 0.01, between WT and the indicated mouse group.

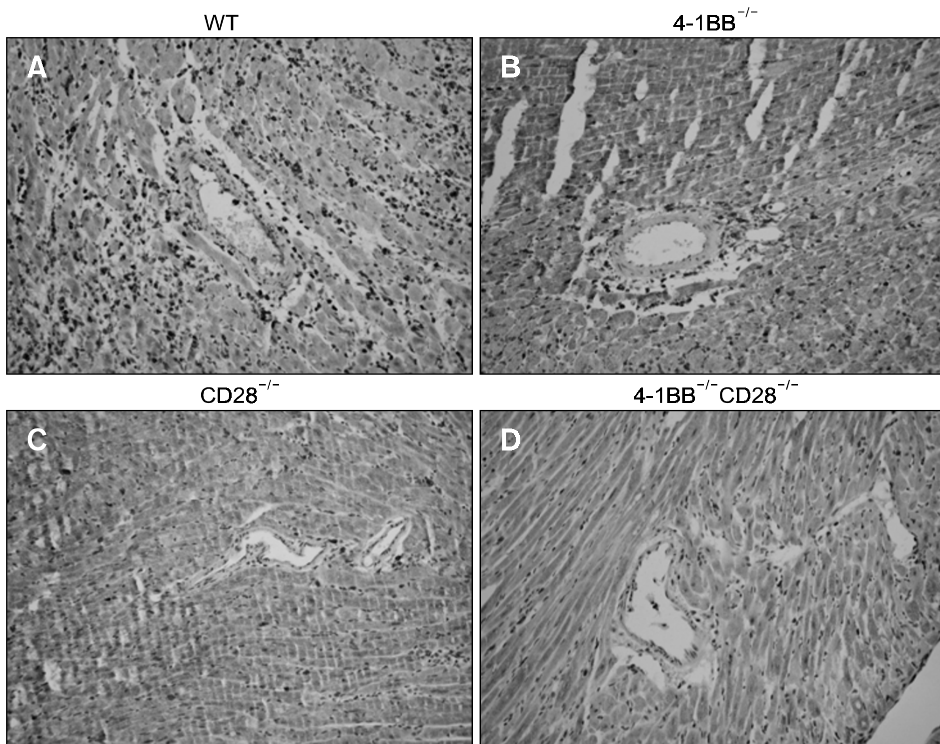


Figure 2. Immunostaining of cardiac allografts. Grafts were harvested at 7 d post transplantation. Sections of frozen allografts were stained with anti-CD3 as described in Materials and Methods.

tation showed that delayed rejection in the deficient mice correlates with less severe T lymphocyte infiltration (Figure 2). Collectively, our results indicate that the long-term survival of cardiac allografts in CD28/4-1BB-deficient mice is due mainly to the absence of CD28 costimulatory signals.

We next performed an *in vitro* MLR to examine the responsiveness of T cells to allostimulation. We stimulated WT, CD28-deficient, 4-1BB- deficient, and CD28/4-1BB-deficient Balb/c T cells with C57BL/6 APCs. There was no difference in the proliferative ability of alloantigen-stimulated T cells of WT and 4-1BB-deficient mice (Figure 3A). In contrast, proliferation of T cells of CD28-deficient and CD28/4-1BB-deficient mice was markedly decreased compared with WT and 4-1BB-deficient mice. Even though the 4-1BB deficiency further decreased the proliferation induced by the CD28 deficiency ($P <$ 0.0049), it seemed that CD28 signals had a greater effect on proliferation than 4-1BB did in CD28/4-1BB-deficient mice (Figure 4A). Consistent with this conclusion, CD28-deficient and CD28/4-1BB-deficient mice had a comparable decrease in the production of IFN-γ and IL-2 by T cells after allostimulation as compared with WT mice (Figure 3B, C). Our results suggest that the proliferation and cytokine secretion of alloreactive T cells is dependent mainly on CD28 signals.

To determine the dependency of the allospecific Ab response on CD28, 4-1BB, or both signals, sera were collected at days 0, 7, 14, 21 and 28 after cardiac transplantation and cells derived from the donor strain (EL4 cells) were stained with the harvested sera. FACS analysis showed that allospecific IgG began to be detected in WT mice at day 14 and its levels reached a peak at day 21 and were decreased thereafter (Figure 4). 4-1BB-deficient mice produced similar levels of allospecific IgG during the first two weeks and thereafter its levels continued to increase and exceeded those of WT mice. In contrast to WT and 4-1BB-deficient mice, both CD28-deficient and CD28/4-1BB-deficient recipients produced basal levels of allospecific IgG (Figure 4). Taken together, our data indicate that the Ab response to alloantigens is dependent largely upon CD28 costimulatory signals. More importantly, the enhanced Ab response in 4-1BB-deficient mice is abolished in CD28/4-1BB-deficient mice, suggesting that 4-1BB signals do not make a significant contribution to the Ab response to alloantigens in the absence of CD28 signals.

Finally, we performed an *in vivo* CTL assay to examine the cytotoxic activity against alloantigens in WT, CD28-deficient, 4-1BB-deficient, and CD28/4-1BB-deficient mice. Figure 5 shows the extent of lysis of donor C57BL/6 splenocytes by Balb/c T cells 4 h after they had been transferred into the recipient. The strong CTL activity against target cells was

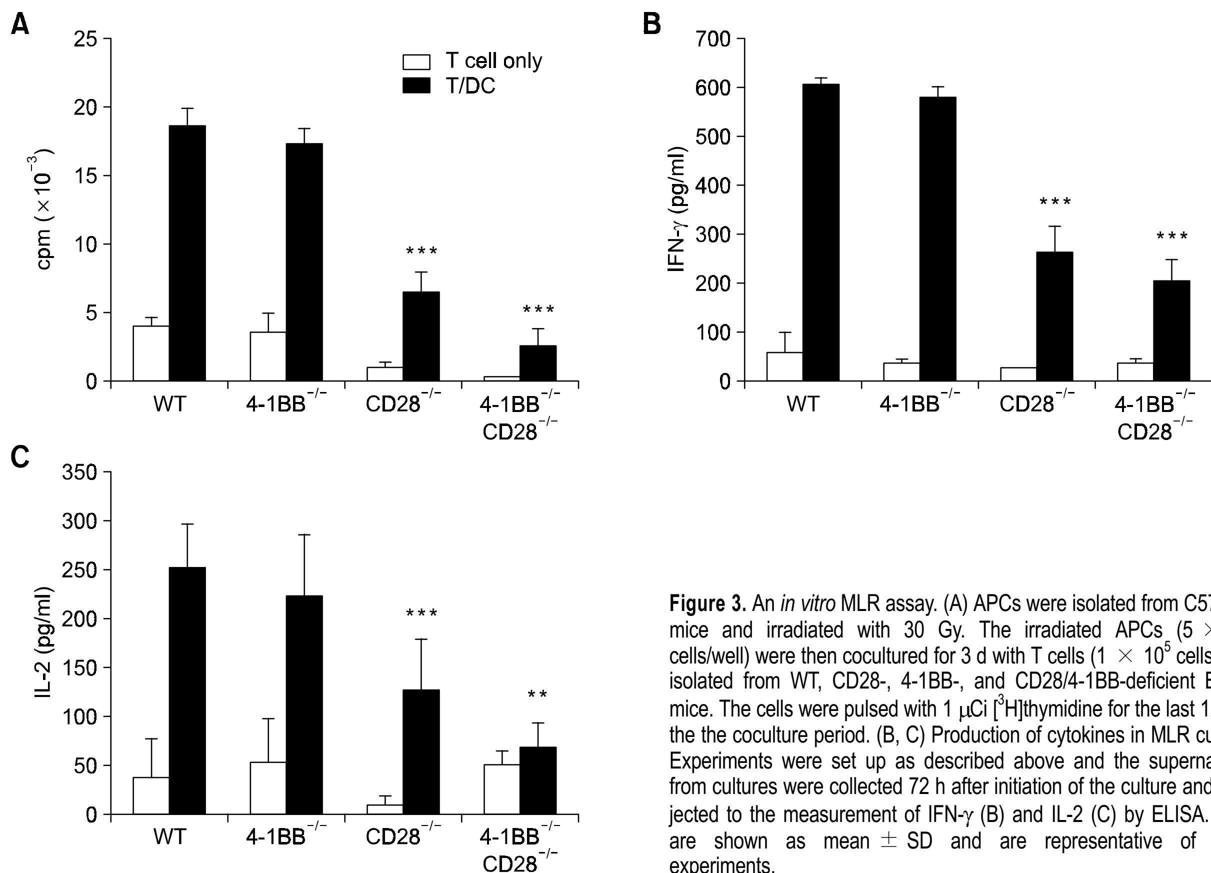


Figure 3. An *in vitro* MLR assay. (A) APCs were isolated from C57BL/6 mice and irradiated with 30 Gy. The irradiated APCs (5×10^5 cells/well) were then cocultured for 3 d with T cells (1×10^5 cells/well) isolated from WT, CD28-, 4-1BB-, and CD28/4-1BB-deficient Balb/c mice. The cells were pulsed with 1 μCi [^{3}H]thymidine for the last 12 h of the the coculture period. (B, C) Production of cytokines in MLR culture. Experiments were set up as described above and the supernatants from cultures were collected 72 h after initiation of the culture and subjected to the measurement of IFN-γ (B) and IL-2 (C) by ELISA. Data are shown as mean ± SD and are representative of three experiments.

observed in WT T cells. Not unexpectedly, 4-1BB-deficient T cells have comparable levels of CTL. CD28-deficient and CD28/4-1BB-deficient T cells had a much lowest CTL activity compared with WT and 4-1BB-deficient mice. These results suggest that, like the antibody response, the role of 4-1BB signals is negligible in the absence of CD28 signals.

Discussion

An important question related to the 4-1BB study is whether 4-1BB costimulation is dependent on CD28 signals. Since 4-1BB has a distinct signaling pathway from CD28 (Saoulli *et al*., 1998), and 4-1BB has a later role in sustaining the immune response (Cannon *et al*., 2001), it is believed that 4-1BB functions independently of CD28. In one model, however, 4-1BB stimulation enhances a cross-priming of CTL to tumor Ags only in the presence of intact CD28 signals (Diehl *et al*., 2002). Presumably, this is due to the requirement for prior upregulation of 4-1BB by both TCR stimulation and CD28 costimulation before 4-1BB functions. In this study, we demonstrated that despite their normal responsive ability to allostimulation (Figure 3), 4-1BB-deficient mice generated a more enhanced Ab and a similar level of CTL response to alloantigens as compared with WT mice (Figures 4 and 5). More importantly, these immune responses of 4-1BB-deficient mice were abrogated in the absence of CD28 signals, suggesting that 4-1BB signals depend heavily upon CD28 signals.

Initial description of 4-1BB-deficient mice has shown that 4-1BB-deficient splenocytes were hyperresponsive with regard to proliferation when stimulated *in vitro* with plate-coated anti-CD3 (Kwon *et al*., 2002b). Recently, Croft's group has extended this observation by showing that 4-1BB-deficient CD4+ T cells have more enhanced responsiveness to a nominal antigen *in vivo*, which is not due to a developmental defect (Lee *et al*., 2005). However, they didn't observe any abnormal Ab response in 4-1BB-deficient mice. Our result seems to be contradictory to the observation by Croft's group in that even though WT and 4-1BB-deficient T cells have a similar extent of proliferation and cytokine secretion after allostimulation, 4-1BB-deficient mice display an enhanced Ab response to alloantigens. There exists several circumstantial evidence sup-

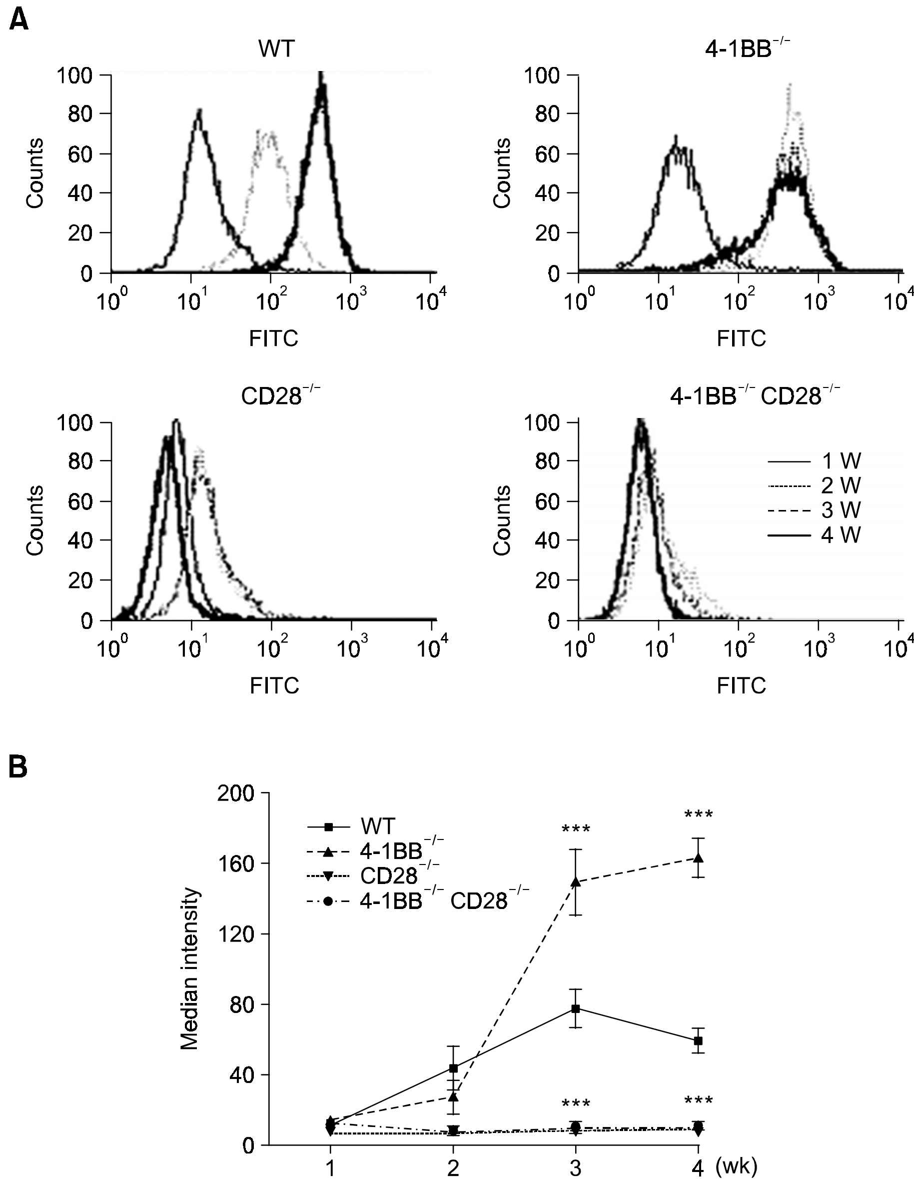
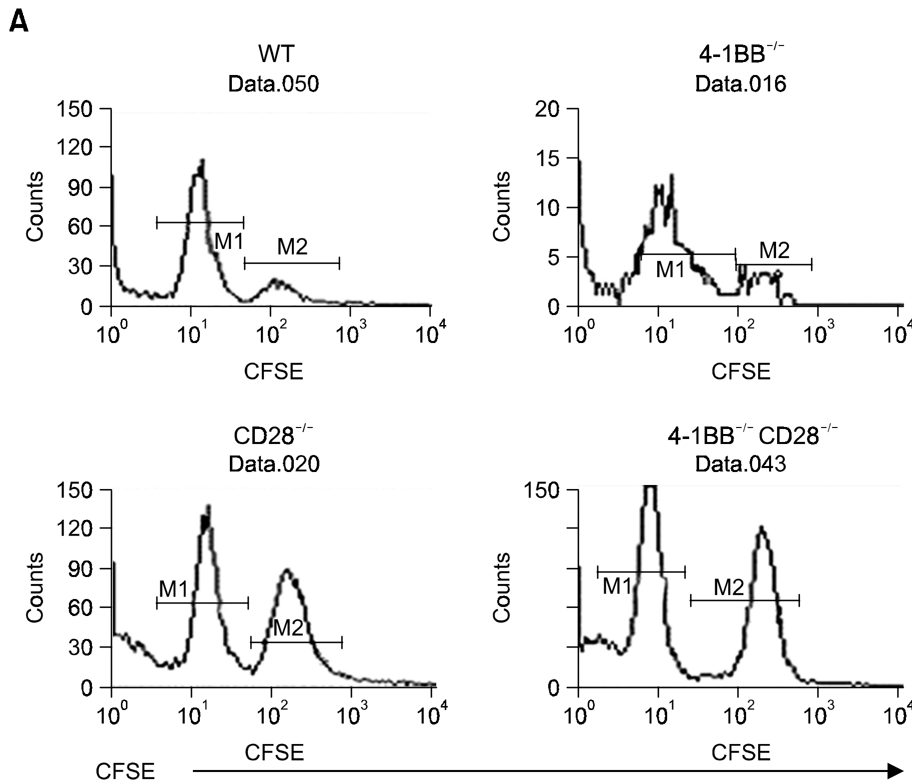


Figure 4. Alloantigen-specific Ab responses. Serum was harvested at weeks 0, 1, 2, 3, and 4 after cardiac transplantation. Levels of alloantigen-specific Ab were quantified by flow cytomery as described in Materials and Methods. (A) Representative FACS histograms. (B) Median intensity. Data are presented as mean ± SEM (n = 5-10 per group). ***$P < 0.001$.

porting a higher Ab response in 4-1BB-deficient mice; 4-1BB-deficient mice have increased basal levels of Ig (Vinay *et al*., 2003). Moreover, blockage of interactions between 4-1BB and 4-1BBL elevates levels of autoantibody in cGVHD (Nozawa *et al*., 2001), whereas stimulation of 4-1BB abrogates T cell-dependent Ab response (Mittler *et al*., 1999; Sun *et al*., 2002; Foell *et al*., 2003; Wu *et al*., 2003; Foell *et al*., 2004; Seo *et al*., 2004; Kim *et al*., 2005) and sustained stimulation of 4-1BB in 4-1BBL transgenic mice induces progressive depletion of B cells and subsequent abrogation of Ab production (Zhu *et al*., 2000). It seems that 4-1BB signals are involved in Th1 immune responses (our unpublished data) and it has been shown that Th1-specific Ab responses are impaired in 4-1BB-deficient mice (Kwon *et al*., 2002b). Conversely, it is possible that Th2-specific Ab responses would be elevated in 4-1BB-deficient mice particularly in conditions that $CD4^+$ T cells are stimulated by strong Ags such as alloantigens (Lee *et al*., 2005). Therefore, we suspect that high levels of Th2-specific isotypes were responsible for the increased Ab response to alloantigens in 4-1BB-deficient mice that we observed in this study (Figure 4).

In vivo ligation of 4-1BB markedly elevate the effector function of $CD8^+$ T cells by increasing their expansion and survival. It also can enhance $CD8^+$ T cell immunity to poor immunogenic Ags (Halstead *et al*., 2002) and break $CD8^+$ T cell tolerance (Wilcox *et*



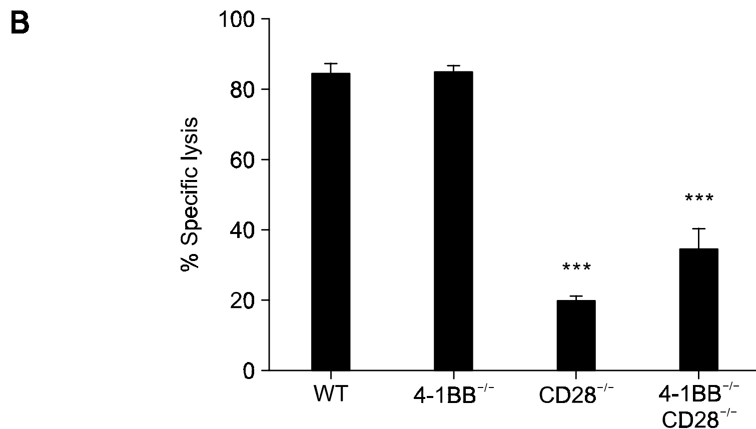


Figure 5. An *in vivo* CTL assay. An *in vivo* CTL assay was performed as described in Materials and Methods. (A) Representative FACS hitograms. (B) Specific lysis. Data are presented as mean ± SEM (n = 5-10 per group). ***P < 0.001.

al., 2002; 2004). Surprisingly, 4-1BB-deficient and 4-1BBL-deficient mice had no or a minor defect in $CD8^+$ T cell activity as compared with CD28-deficient mice (DeBenedette *et al*., 1999; Tan *et al.,* 1999; Kwon *et al*., 2002b). Our present data are consistent with this observation. However, it is not known why a discrepancy exists depending on experimental models used. Collectively, our results reveal 4-1BB as a negative regulator for some aspects of alloimmune responses.

It is surprising that there was a significantly prolonged survival of cardiac allografts in 4-1BB-deficient mice as compared with WT mice, provided that these knockout mice have enhanced general immune responses to alloantigens. Further studies will be needed to solve this puzzling problem. Overall, the present study suggests that since 4-1BB signaling is dependent upon CD28 signaling, combined blockage of the two costimulatory molecules may not be an effective way to induce tolerance to allografts.

The dependency of 4-1BB signals on the CD28 costimulatory pathway implies that 4-1BB plays a role downstream of CD28 signals in the process of T cell activation. This may be because high levels of

4-1BB expression on T cells require TCR (T-cell receptor) triggering in combination with CD28 costimulation (Deihl *et al.*, 2002). Provision of signals through 4-1BB results in increased survival and expansion of Ag-primed T cells (Cooper *et al.*, 2002). Even though, in this study, we provided a possibility that 4-1BB signals depends on CD28 signals, we don't know the mechanism underlying our observations. Further studies will be needed to obtain a conclusive result regarding the hierarchy of costimulatory molecules, including 4-1BB and CD28.

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