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Requirement for Donor and Recipient CD40 Expression in Cardiac Allograft Rejection: Induction of Th1 Responses and Influence of Donor-Derived Dendritic Cells[1]

Meera J. Nathan,[†] Jeffrey E. Mold,[*] Sherri C. Wood,[*] Keri Csencsits,[*] Guanyi Lu,[*] Ernst J. Eichwald,[‡] and D. Keith Bishop[2*†]

Costimulation through the CD40-CD40 ligand (CD40L) pathway is critical to allograft rejection, in that anti-CD40L mAb therapy prolongs allograft survival. However, the majority of studies exploring CD40-CD40L interactions have targeted CD40L. Less is known about the requirement for donor- and/or host-derived CD40 during rejection. This study assessed the relative contributions of donor and recipient CD40 expression to the rejection process. As the effectiveness of costimulatory blockade may be mouse strain dependent, this study explored the requirement for donor and recipient CD40 expression in BALB/c and C57BL/6 mice. Wild-type (WT) and CD40$^{-/-}$ BALB/c recipients readily rejected WT and CD40$^{-/-}$ C57BL/6 allografts, and rejection was associated with a prominent Th1 response. In contrast, CD40$^{-/-}$ C57BL/6 recipients failed to reject WT or CD40$^{-/-}$ BALB/c allografts and did not mount Th1 or Th2 responses. However, injection of donor CD40$^{-/-}$ dendritic cells induced both Th1 and Th2 responses and allograft rejection in CD40$^{-/-}$ C57BL/6 recipients. Finally, WT C57BL/6 mice rejected CD40$^{-/-}$ allografts, but this rejection response was associated with muted Th1 responses. These findings demonstrate that 1) CD40 expression by the recipient or the graft may impact on the immune response following transplantation; 2) the requirement for CD40 is influenced by the mouse strain; and 3) the requirement for CD40 in rejection may be bypassed by donor DC. Further, as CD40 is not required for rejection in BALB/c recipients, but anti-CD40L mAb prolongs graft survival in these mice, these results suggest that anti-CD40L therapy functions at a level beyond disruption of CD40-CD40L interactions. ***The Journal of Immunology*****, 2004, 172: 6626–6633.**

As T cells are the primary mediators of allograft rejection (1, 2), much effort has been directed at designing therapeutics that specifically block the initial activation of T cells in allograft recipients. T cell activation is a multistep process requiring two signals for activation (3). The first signal results from the engagement of the TCR with the MHC-peptide complex on the APC, followed by a second costimulatory signal resulting from the engagement of cell surface markers on the T cell and APC. Absence of the second signal results in either T cell anergy (3) and/or deletion of these cells (4, 5).

The CD28-CD80/86 and CD40-CD40 ligand (CD40L)[3] pathways are two well-characterized costimulatory pathways. Both pathways are critical in the activation and maturation of both T cells and APC, and thus have been explored as therapeutic targets in a number of transplant models (reviewed in Refs. 6 and 7). Further, these two costimulatory pathways are intimately linked in the activation of APC, B cells, and T cells. Although CD40L expression is primarily restricted to activated CD4$^+$ and CD8$^+$ T cells, mast cells, and activated platelets, CD40 is constitutively expressed on APC and other cell types, including macrophages, dendritic cells (DC), B cells, endothelial cells (EC), and fibroblasts (reviewed in Refs. 8 and 9). When CD40L on T cells interacts with CD40 on APC, APC up-regulate the expression of CD80 and CD86. Subsequently, CD80/86 on the activated APC deliver costimulatory signals through CD28 on the T cell. Hence, disruption of the CD40-CD40L pathway is thought to prevent T cells from receiving the requisite costimulatory signal through CD28, thereby rendering the T cells anergic (3). In addition to this APC-activating process, a growing body of evidence indicates that cross-linking CD40L may directly deliver signals to the T cell that may influence the profile of cytokines produced as well as induce apoptosis of the T cell (10–13). Indeed, cross-linking CD40L on T cells has been reported to activate several signal transduction molecules, including c-Jun N-terminal kinase-p38 pathways and induction of neutral sphingomyelinase (14, 15).

The majority of studies exploring the CD40-CD40L pathway in transplantation have used anti-CD40L mAb or CD40L$^{-/-}$ mice (6, 7). Indeed, very little is known regarding the requirement for recipient and/or graft CD40 in the rejection process. In addition to CD40's roles in T cell, B cell, and APC activation, engagement of CD40 on EC results in the up-regulation of cell surface adhesion molecules such as CD62E, VCAM-1, and ICAM, which are required for leukocyte recruitment into tissues (16–19). Hence, perturbation of CD40 expressed by the endothelium of the graft may facilitate leukocyte infiltration during the rejection process. Further, although it is known that CD40$^{-/-}$ mice have impaired Ig class switching (20, 21), the role of alloantibodies in the rejection process has yet to be completely defined (22–25). To explore these potential roles for CD40 in allograft rejection, this study compared

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[3] Abbreviations used in this paper: CD40L, CD40 ligand; DC, dendritic cell; EC, endothelial cell; MST, mean survival time; WT, wild type.

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WT and CD40$^{-/-}$ mice as both cardiac allograft donors and recipients. Finally, as the effectiveness of costimulatory blockade has been reported to be dependent in part on the mouse strain used (26), CD40$^{-/-}$ BALB/c and C57BL/6 mice were used as allograft donors and recipients. Our findings document that CD40$^{-/-}$ BALB/c mice acutely reject CD40$^{-/-}$ C57BL/6 cardiac allografts, and that graft rejection is associated with the induction of a potent Th1 response. In contrast, CD40$^{-/-}$ C57BL/6 recipients fail to mount Th1 responses and do not reject CD40$^{-/-}$ BALB/c allografts; this requirement for CD40 may be overridden by injection of recipients with CD40$^{-/-}$ BALB/c DC. These observations point to further differences in targeting CD40 vs CD40L and emphasize the complexity of targeting the CD40-CD40L costimulatory pathway as a therapeutic modality. Further, these findings document that the potent immunostimulatory capacity of DC is independent of CD40 expression.

Materials and Methods

Mice

Female WT C57BL/6, BALB/c, and C3H/He mice were obtained from Charles River Breeding Laboratories (Raleigh, NC). Breeder pairs of CD40$^{-/-}$ C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeder pairs of CD40$^{-/-}$ BALB/c mice were provided by Dr. R. Noelle (Dartmouth College, Lebanon, NH). Colonies of CD40$^{-/-}$ mice were established in the rodent facilities maintained by the Unit for Laboratory Animal Medicine at University of Michigan. CD40 deficiency is routinely verified by flow cytometry. Mice for this study were used between 6 and 12 wk of age.

Media

The culture medium used in these studies was DMEM supplemented with 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 mM folic acid, 5×10^{-5} M 2-ME (all obtained from Sigma-Aldrich, St. Louis, MO), 1.6 mM L-glutamine, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 2% FCS (all obtained from Life Technologies, Grand Island, NY).

Heterotopic cardiac transplantation

C57BL/6 (H-2^b) or BALB/c (H-2^d) mice were transplanted with intact BALB/c or C57BL/6 cardiac allografts respectively, as previously described (27). Where indicated, CD40$^{-/-}$ C57BL/6 and CD40$^{-/-}$ BALB/c mice were used as allograft recipients and/or donors. In this model, the donor heart is anastomosed to the great vessels of the abdomen, perfused with recipient mouse's blood, and resumes contraction. Transplant function was monitored by daily abdominal palpation, and graft rejection was indicated by cessation of contractions. Histologic evidence of rejection (i.e., leukocytic infiltration, loss of myocyte nuclei, and cross-striation) was verified by H&E staining of formalin-fixed allograft fragments.

Assessment of primed Th1 function

To monitor primed Th1 function, splenocytes (1×10^6 cells/ml) isolated from allograft recipients were stimulated with irradiated (5000 rad) donor strain splenocytes (1×10^6 cells/ml) for 72 h, and the concentration of IFN-γ in the culture supernatant was measured by ELISA (see below). This assay detects in vivo primed Th1, in that splenocytes from naive, nontransplanted mice produce minimal or undetectable concentrations of IFN-γ under these conditions (28, 29).

IFN-γ ELISA

Experimental samples (100 μl) were added to ELISA plate wells coated with 5 μg/ml rat anti-mouse IFN-γ capture Ab (clone R4-6A2; BD PharMingen, San Diego, CA). After a 1-h incubation at room temperature, plates were washed with Tween 20 (0.05%) in PBS and incubated with 100 μl of rat anti-mouse IFN-γ biotinylated Ab (clone XMG1.2; 1 μg/ml; BD PharMingen) at room temperature for 45 min. Plates were then washed, and 100 μl of 1/600 diluted avidin-peroxidase (Sigma-Aldrich) was added. After a 30-min incubation at room temperature, plates were developed with ABTS substrate (Sigma-Aldrich). After 20 min, absorbance was determined at 405 nm by an EL 800 microtiter plate reader (Bio-Tek Instruments, Winooski, VT). Sample cytokine concentrations were calculated from a standard curve that was derived from 2-fold serial dilutions of recombinant mouse IFN-γ, and data are reported as the mean $\pm$ SEM of IFN-γ production for 3 to 11 individual mice per experimental group.

ELISPOT assay for IFN-γ- and IL-4-producing cells

The ELISPOT assay used to quantify alloantigen-primed cytokine producing cells has been described previously (30). Capture and detection mAbs were obtained from BD PharMingen. Polyvinylidene fluoride-bottom plates (Millipore, Bedford, MA) were coated overnight with rat anti-mouse IFN-γ (R4-6A2; 4 μg/ml) or IL-4 (11B11; 2 μg/ml) capture Abs, blocked for 90 min with 1% BSA in PBS at room temperature, and washed three times with PBS. Irradiated (5000 rad) donor strain splenocytes (4×10^5) were added to each well, followed by 1×10^6 splenocytes obtained from allograft recipients. Plates were incubated for 24 h at 37°C, then washed three times with PBS, followed by four times with PBS-Tween 20 (0.05%). One hundred microliters of biotinylated rat anti-mouse mouse IFN-γ (XMG1.2; 2 μg/ml) or IL-4 (BVD6-24G2; 2 μg/ml) detection Abs were added to each well, and incubated overnight at 4°C. Plates were washed three times with PBS-Tween 20, then 100 μl of alkaline phosphatase-conjugated anti-biotin Abs (1/1000 dilution; Vector Laboratories, Burlingame, CA) were added to the IFN-γ wells, and 100 μl of HRP-conjugated streptavidin (1/2000 dilution; DAKO, Carpinteria, CA) was added to the IL-4 wells for 90 min at room temperature. Plates were washed four times with PBS, developed with nitro blue tetrazolium/BCIP or 3-amino-9-ethylcarbozole, washed with H_2O, and air-dried. Spots were enumerated using an ImmunoSpot series 1 ELISPOT Analyzer (Cellular Technologies, Cleveland, OH).

Generation of donor-derived, third-party, and syngeneic DC for injection into CD40$^{-/-}$ C57BL/6 allograft recipients

As previously described (31), bone marrow was isolated from the femurs and tibias of CD40$^{-/-}$ BALB/c (donor strain), WT C3H/He (third-party), or CD40$^{-/-}$ C57BL/6 (syngeneic) mice by flushing with HBSS. Whole bone marrow cells were subjected to hypotonic lysis to remove RBC, and the remaining cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 culture medium containing 10 ng/ml GM-CSF and IL-4 (PeproTech, Rocky Hill, NJ). Cells were incubated at 37°C in 10% CO_2 for 72 h, at which point the nonadherent cells were removed, washed, and resuspended in fresh culture medium supplemented with fresh GM-CSF and IL-4 and incubated for 48 additional hours. After incubation, the nonadherent cells were removed and suspended in fresh culture medium at a concentration of 5×10^6 cells/ml, layered over a 14.5% metrizamide (Sigma-Aldrich) gradient in an equivalent volume, and subjected to centrifugation at 2000 rpm for 15 min at 4°C. Isolated DC were harvested from the central monolayer and washed three times to remove excess metrizamide and an additional three times in PBS before i.v. injection into cardiac allograft recipients. The purity of the DC, as measured by surface expression of CD11c and class II, was ~80%. CD40$^{-/-}$ C57BL/6 recipients of CD40$^{-/-}$ BALB/c cardiac allografts were injected i.v. with 2.5×10^6 CD40$^{-/-}$ BALB/c, WT C3H/He, or CD40$^{-/-}$ C57BL/6 DC the day before transplantation.

Statistical analysis

The p values were obtained by ANOVA analysis and a Fisher's protected least significant difference post-hoc test. All statistical analyses were performed using StatView 5.0.1 software (SAS Institute, Cary, NC).

Results

CD40 expression is not required for allograft rejection in BALB/c recipients

To determine the impact of CD40 expression on allograft rejection, WT or CD40$^{-/-}$ BALB/c recipients were transplanted with either WT or CD40$^{-/-}$ C57BL/6 cardiac allografts. WT BALB/c recipients readily rejected WT and CD40$^{-/-}$ allografts with mean survival times (MST) of 10.3 and 9.6 days, respectively (Fig. 1). Similarly, CD40$^{-/-}$ BALB/c recipients rejected both WT and CD40$^{-/-}$ allografts, although rejection of CD40$^{-/-}$ allografts occurred in a slightly delayed fashion (MST, 12.1 days for WT grafts vs 14.3 days for CD40$^{-/-}$ grafts; $p = 0.0042$), suggesting that expression of CD40 by the graft may contribute to the rejection process. Nonetheless, histologic analyses of both WT and CD40$^{-/-}$ allografts revealed similar patterns of rejection, which were characterized by an intense mononuclear cellular infiltrate, many dead and degenerating myocytes, and inflamed arteries (data not shown). Hence, the expression of CD40 by the recipient and/or graft was not required for acute allograft rejection in BALB/c mice.

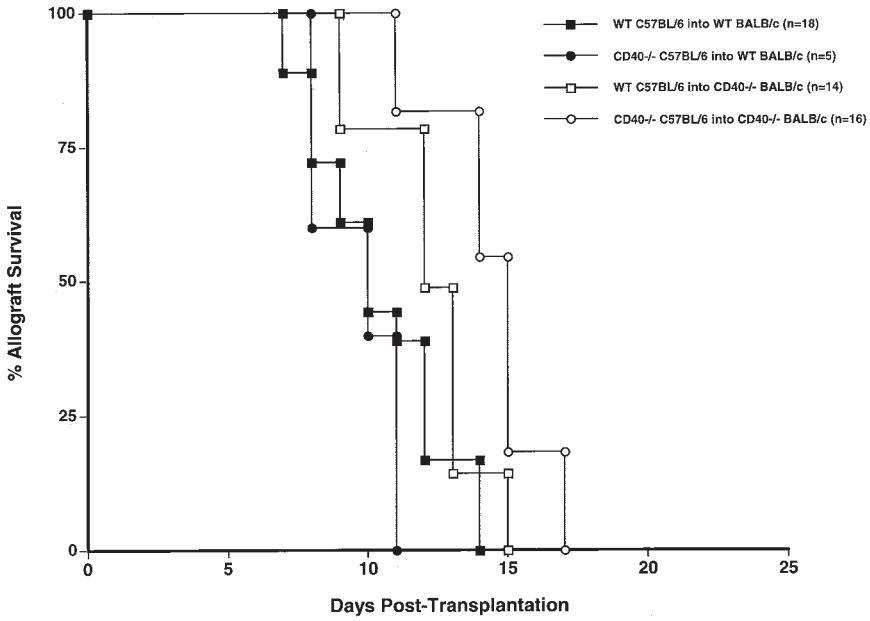

FIGURE 1. BALB/c recipients reject cardiac allografts regardless of CD40 expression. WT and CD40$^{-/-}$ BALB/c mice were transplanted with either WT or CD40$^{-/-}$ C57BL/6 allografts as indicated. The number of transplants per experimental group is given in parentheses, and transplant function was evaluated by daily palpation.

In vivo priming of IFN-γ-producing Th1 in BALB/c allograft recipients

In this model unmodified allograft rejection is associated with a dominant Th1 response (32). Hence, splenocytes obtained from BALB/c allograft recipients were stimulated with donor strain splenocytes for 72 h and assessed for IFN-γ release as a measure of in vivo Th1 priming (28, 29). Splenocytes obtained from naive, nontransplanted WT or CD40$^{-/-}$ BALB/c mice produced minimum IFN-γ upon stimulation with alloantigens (Fig. 2). In contrast, splenocytes obtained from all experimental groups produced significant levels of IFN-γ relative to their naive controls, with the level of significance ranging from $p = 0.0048$ to $p = 0.0003$. Although it appeared that WT BALB/c recipients of CD40$^{-/-}$ allografts exhibited less Th1 priming than WT recipients of WT grafts, this difference did not reach statistical significance ($p = 0.3533$). Hence, donor and/or recipient CD40 expression was not required for Th1 priming after transplantation in BALB/c mice.

CD40 expression is required for allograft rejection in C57BL/6 recipients

We next evaluated the requirement for CD40 expression in C57BL/6 mice, as Williams et al. (26) reported that sensitivity to costimulatory blockade after allogeneic skin grafting is mouse strain dependent. WT C57BL/6 recipients acutely rejected both WT and CD40$^{-/-}$ BALB/c cardiac allografts, with rejection of CD40$^{-/-}$ grafts (MST, 9.6 days) being slightly delayed relative to that of WT allografts (MST, 7.7 days; $p = 0.0171$). Interestingly, CD40$^{-/-}$ C57BL/6 recipients were incapable of acutely rejecting either WT or CD40$^{-/-}$ allografts, indicating that CD40 expression by the recipient was critical to the rejection process in C57BL/6 mice. Note that the arrows in Fig. 3 indicate that allografts placed in CD40$^{-/-}$ C57BL/6 mice were functioning at the time of harvest. Hence, the vast majority of these allografts continued to function normally for at least 40 days. Histologic analyses of functioning allografts harvested between days 50 and 60 revealed markedly preserved cardiac architecture, with viable myocytes and only scattered areas of diffuse infiltrate (data not shown).

In vivo priming of IFN-γ producing Th1 in C57BL/6 allograft recipients

As described in Fig. 2 for BALB/c recipients, splenocytes from C57BL/6 allograft recipients were assessed for primed Th1 function, with naive WT and CD40$^{-/-}$ C57BL/6 splenocytes serving as negative controls. As shown in Fig. 4, WT C57BL/6 recipients

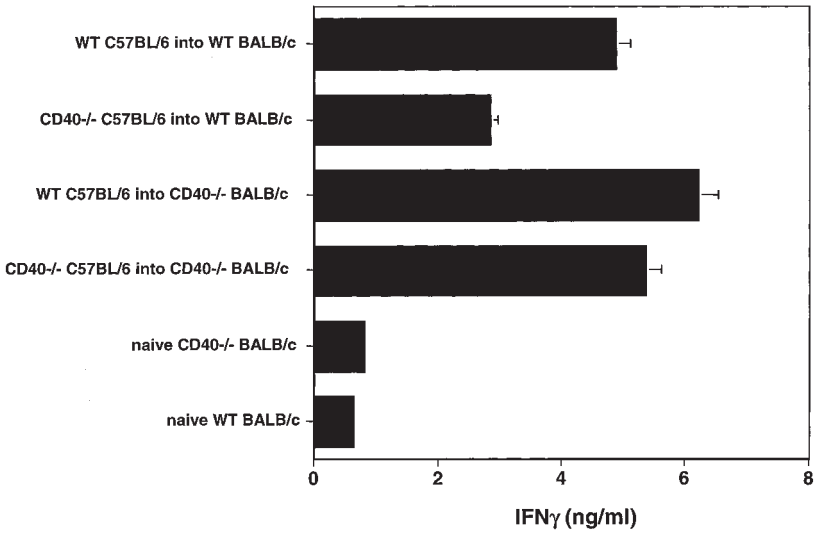

FIGURE 2. Th1 priming in BALB/c cardiac allograft recipients. WT and CD40$^{-/-}$ BALB/c mice were transplanted with either WT or CD40$^{-/-}$ C57BL/6 allografts as indicated. Recipient splenocytes were harvested at the time of allograft rejection and stimulated with irradiated donor strain splenocytes in MLC for 72 h. Supernatants were collected, and IFN-γ concentrations were determined by ELISA as a measure for Th1 priming. Naive WT and CD40$^{-/-}$ BALB/c splenocytes obtained from nontransplanted mice were stimulated in MLC and served as negative controls for Th1 priming. Data are reported as the mean ± SEM of IFN-γ production obtained from 3 to 11 individual animals per experimental group.

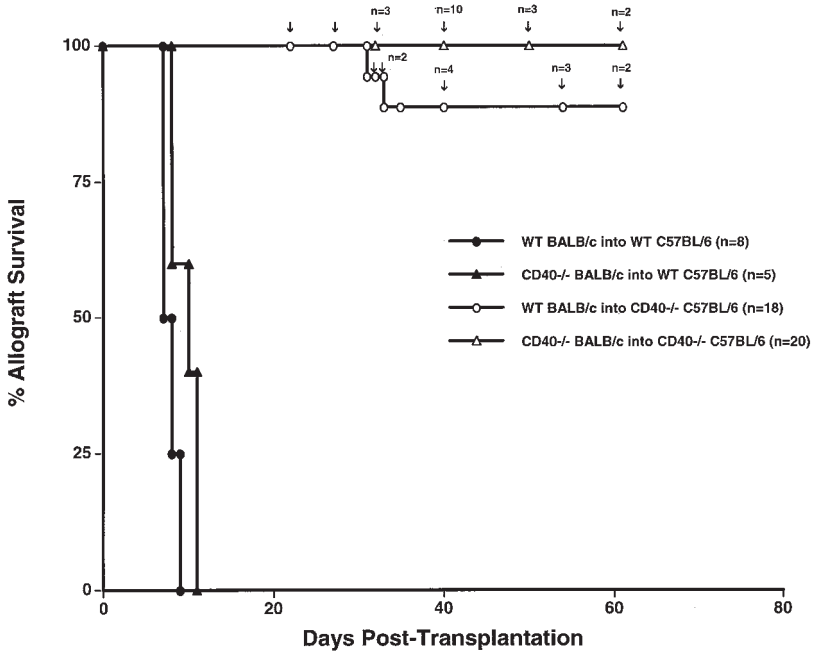

FIGURE 3. CD40 expression is required for cardiac allograft rejection in C57BL/6 recipients. WT and CD40$^{-/-}$ C57BL/6 mice were transplanted with either WT or CD40$^{-/-}$ BALB/c allografts as indicated. The number of transplants per experimental group is given in parentheses, and transplant function was evaluated by daily palpation. Arrows indicate the days that functioning transplants were harvested for immunologic assays, with the number of functioning grafts that were harvested at a given time point noted by the arrows.

of WT allografts mounted strong Th1 responses ($p < 0.0001$ compared with naive controls). Interestingly, although WT C57BL/6 mice acutely rejected CD40$^{-/-}$ allografts, primed Th1 function was markedly reduced compared with that observed for WT recipients of WT allografts ($p = 0.0073$). This observation suggests that expression of CD40 by allograft impacts on Th1 priming, although this does not markedly alter the eventual rejection of the graft (Fig. 3). CD40$^{-/-}$ C57BL/6 recipients, which failed to reject either WT or CD40$^{-/-}$ allografts, also did not mount Th1 responses to the transplant (Fig. 4). Collectively, these observations indicate that both donor and recipient expression are required for optimal Th1 priming in C57BL/6 mice, and that recipient expression of CD40 is essential for acute allograft rejection.

ELISPOT analysis for cytokine production in CD40$^{-/-}$ allograft recipients

Costimulatory blockade has been reported to inhibit Th1, yet spare Th2, responses (33), although this is not a universal observation (reviewed in Ref. 9). Hence, we used a sensitive ELISPOT assay (30) to quantify the frequency of primed IFN-γ-producing Th1 and IL-4-producing Th2 in CD40$^{-/-}$ recipients of CD40$^{-/-}$ allografts (Fig. 5). In the complete absence of CD40, BALB/c mice mounted significant Th1 responses compared with their C57BL/6 counterparts ($p < 0.0001$), verifying that these two mouse strains have distinct requirements for CD40 in Th1 responsiveness to an allograft. Although the effect was less prominent, CD40$^{-/-}$ BALB/c mice also mounted greater Th2 responses to CD40$^{-/-}$ allografts than did their CD40$^{-/-}$ C57BL/6 counterparts ($p = 0.0167$). Hence, CD40 deficiency did not lead to preferential Th2 skewing in either setting. It should be noted that naive CD40$^{-/-}$ splenocytes from either recipient strain produced negligible IFN-γ or IL-4 under these conditions (data not shown).

Donor-derived CD40$^{-/-}$ DC induce allograft rejection and T cell responses in CD40$^{-/-}$ C57BL/6 recipients

We have found that CD40 expression by DC is not required to stimulate allogeneic T cell responses in vitro and in vivo (J. E.

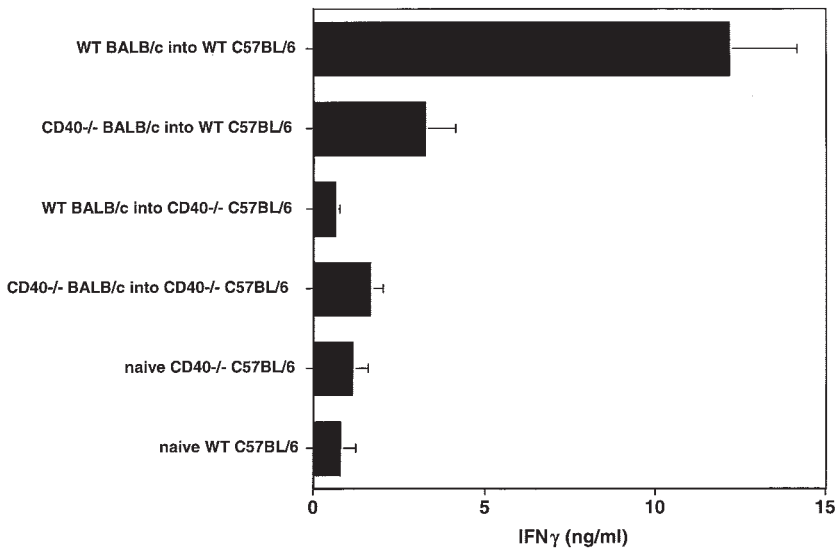

FIGURE 4. Th1 priming in C57BL/6 cardiac allograft recipients. WT and CD40$^{-/-}$ C57BL/6 mice were transplanted with either WT or CD40$^{-/-}$ BALB/c allografts as indicated. Recipient splenocytes were harvested either at the time of allograft rejection (for WT recipients) or at the termination of the experiment (for CD40$^{-/-}$ recipients, which did not reject their grafts) and were stimulated with irradiated donor strain splenocytes in MLC for 72 h. Supernatants were collected, and IFN-γ concentrations were determined by ELISA as a measure of Th1 priming. Naive WT and CD40$^{-/-}$ C57BL/6 splenocytes obtained from nontransplanted mice were stimulated in MLC and served as negative controls for Th1 priming. Data are reported as the mean ± SEM of IFN-γ production and were obtained from 4 to 11 individual animals per experimental group.

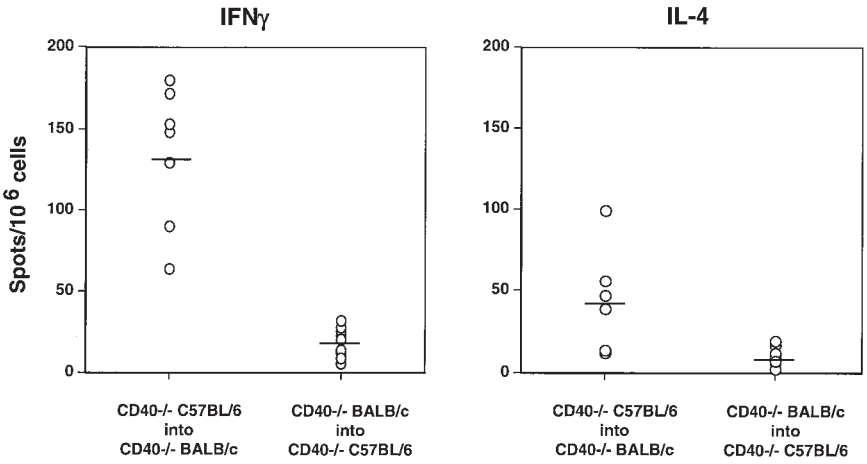

FIGURE 5. ELISPOT analysis of Th1 and Th2 responses in CD40$^{-/-}$ cardiac allograft recipients. CD40$^{-/-}$ BALB/c or C57BL/6 were transplanted with CD40$^{-/-}$ C57BL/6 or BALB/c cardiac allografts, respectively. At the time of rejection (CD40$^{-/-}$ BALB/c recipients) or at the termination of the experiment (CD40$^{-/-}$ C57BL/6 recipients), splenocytes were harvested and stimulated with irradiated donor splenocytes in ELISPOT cultures to quantify IFN-γ- and IL-4-producing cells. Data are presented as the number of spot-forming cells per 10^6 splenocytes. The responses of individual animals are plotted ($n = 7$ for CD40$^{-/-}$ BALB/c and $n = 9$ for CD40$^{-/-}$ C57BL/6 recipients). The horizontal bars represent the mean values for each experimental group.

Mold and D. K. Bishop, unpublished observation). This may be due to the fact that propagation of bone marrow derived DC induces some degree of DC maturation, as evidenced by the expression of CD80 and CD86 (34). Indeed, CD40$^{-/-}$ DC express readily detectable levels of CD80 and CD86, which may be increased by incubation of the DC with LPS (data not shown). Hence, we asked whether injection of donor-derived CD40$^{-/-}$ DC would induce rejection of CD40$^{-/-}$ BALB/c allografts in CD40$^{-/-}$ C57BL/6 mice (Fig. 6). One day before transplantation, CD40$^{-/-}$ C57BL/6 recipients of CD40$^{-/-}$ BALB/c cardiac allografts were injected i.v. with 2.5×10^6 CD40$^{-/-}$ BALB/c bone marrow-derived DC or were left untreated. As shown in Fig. 3, untreated CD40$^{-/-}$ recipients retained their CD40$^{-/-}$ allografts until the termination of the experiment (day 30), whereas CD40$^{-/-}$ recipients that received DC injection acutely rejected their CD40$^{-/-}$ allografts (MST, 9.7 days; Fig. 6). To determine the antigenic specificity of DC-induced rejection in these mice CD40$^{-/-}$ recipients of CD40$^{-/-}$ allografts were injected with syngeneic CD40$^{-/-}$ C57BL/6 DC or third-party WT C3H/He DC. Syngeneic DC did not induce the rejection of CD40$^{-/-}$ allografts, indicating that DC expression of CD80 and CD86 alone was not sufficient to facilitate a rejection response. Injection of third-party DC induced rejection in 60% of allograft recipients, but rejection was significantly delayed relative to that observed in recipients of donor-derived DC ($p = 0.0001$).

ELISPOT analyses were used to quantify Th1 and Th2 responses in DC-injected and untreated CD40$^{-/-}$ recipients (Fig. 7). Injection of CD40$^{-/-}$ C57BL/6 recipients with CD40$^{-/-}$ donor-derived DC-induced Th1 (mean number of IFN-γ spots, 202 vs 16 for untreated recipients; $p = 0.0126$) as well as Th2 (mean number of IL-4 spots, 163 vs 19 for untreated recipients; $p = 0.0021$). Thus, in the complete absence of CD40, donor-derived DC were capable of inducing T cell responses and allograft rejection that were otherwise CD40 dependent. As expected, injection of syngeneic DC failed to induce donor-reactive T cell responses. Further, injection of third-party DC failed to induce donor-reactive T cell responses in the majority of recipients ($p = 0.001$ for Th1 and $p < 0.0001$ for Th2). It should be noted that the three recipients of third-party DC that had detectable, albeit low, donor-reactive Th1 responses rejected their allografts. In contrast, recipients of third-party DC that failed to reject their allografts did not mount detectable donor-reactive Th1 responses. As a positive control for the immunogenicity of third-party DC, recipients were assessed for third-party-reactive T cells (Fig. 7, third-party assay). Unlike donor-reactive T cell responses, third-party-reactive Th1 and Th2 response were readily detectable in allograft-bearing recipients of third-party DC.

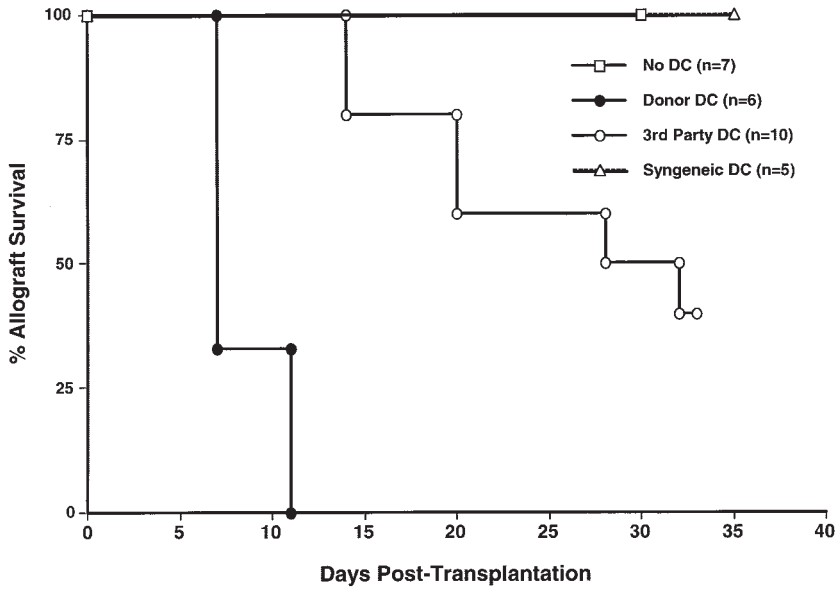

FIGURE 6. Donor-derived CD40$^{-/-}$ DC induce acute allograft rejection in CD40$^{-/-}$ C57BL/6 mice. CD40$^{-/-}$ BALB/c, CD40$^{-/-}$ C57BL/6, and WT C3H/He DC were generated by culturing bone marrow cells in GM-CSF and IL-4 and isolated by metrizamide gradient centrifugation (see *Materials and Methods*). The day before transplantation with CD40$^{-/-}$ BALB/c cardiac allografts, CD40$^{-/-}$ C57BL/6 recipients were injected i.v. (tail vein) with 2.5×10^6 of the indicated population of DC suspended in 200 μl of PBS. Transplant function was monitored by daily palpation. Control allograft recipients that did not receive DC or that were injected with syngeneic DC were sacrificed 30 or 35 days post-transplantation with functioning allografts. Recipients of third-party C3H/He DC were sacrificed at the time of rejection or on day 35 with functioning allografts.

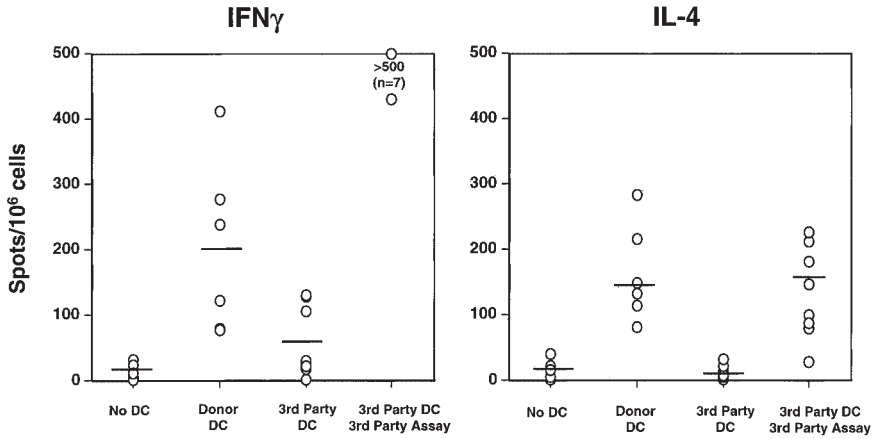

FIGURE 7. Donor-derived CD40$^{-/-}$ DC stimulate Th1 and Th2 responses in CD40$^{-/-}$ C57BL/6 cardiac allograft recipients. Spleens of CD40$^{-/-}$ C57BL/6 transplant recipients described in Fig. 6 were processed into single-cell suspensions and stimulated with irradiated CD40$^{-/-}$ BALB/c splenocytes in ELISPOT cultures to quantify donor-reactive IFN-γ- and IL-4-producing cells. Where indicated (third-party assay), irradiated third-party C3H/He splenocytes were used to stimulate the ELISPOT cultures. Spleens of allograft recipients were harvested either at the time of allograft rejection or at the termination of the experiment. Data are presented as the number of spot-forming cells per 10^6 splenocytes. The responses of individual animals are plotted ($n = 5$ recipients that were not injected with DC, $n = 6$ for recipients that were injected with donor-derived CD40$^{-/-}$ DC, and $n = 8$ for recipients that were injected with third-party DC). The horizontal bars represent the mean values for each experimental group.

Discussion

Disruption of the CD40-CD40L pathway has been established as an effective means to prolong allograft survival (35–40; reviewed in Refs. 6, 7, and 13). However, the majority of these studies have disrupted the pathway by targeting CD40L with anti-CD40L mAb or the use of CD40L$^{-/-}$ mice. Indeed, the contributions of CD40 vs CD40L to the rejection process have not been clearly defined. The use of anti-CD40L mAb is frequently referred to as a blockade of the CD40-CD40L pathway, implying a disruption of the physical interactions between these molecules. However, at least one report (41) provides evidence to support the idea that long-term allograft survival after anti-CD40L therapy is dependent upon complement-mediated depletion of CD40L-expressing cells. A recent report by Monk et al. (42) indicates that the Fc portion of anti-CD40L mAb plays a role in the removal of activated, CD40L-expressing T cells. Further, Blair et al. (12) reported that cross-linking CD40L in conjunction with suboptimal anti-CD3 mAb results in short term T cell activation, the release of IL-10, IFN-γ, and TNF-α, and subsequent apoptosis of the T cells. Indeed, contracting the size of the graft-reactive T cell pool is believed to play an important role in the eventual acceptance of allografts (4, 5). Hence, depletion of graft-reactive, CD40L-expressing T cells by anti-CD40L-induced, complement-mediated lysis or apoptosis would facilitate graft acceptance. In addition, cross-linking CD40L has been shown to increase the production of IL-4 and IL-10 by human T cells (11), and deviation to a Th2 profile of cytokines has been associated with long term graft acceptance after disruption of the CD80/86-CD28 costimulatory pathway (33). However, the once widely held concept that preferential induction of Th2 cytokines may be protective in the context of transplantation has been questioned (reviewed in Ref. 43), and numerous reports have documented that preferential induction of Th2 results in graft rejection (32, 44–48).

CD40 is expressed on a wide variety of cell types, and the consequences of engaging CD40 vary depending upon the responding cell (reviewed in Refs. 8, 9, and 13). For example, CD40 engagement on resting B cells induces Ig isotype switching (49), proliferation (50), cytokine receptor expression (51), and the expression of CD80/86, thereby promoting APC function (52, 53). CD40 perturbation on DC also up-regulates CD80/86 and enhances APC function as well as stimulates the release of IL-12, TNF-α, and various chemokines (9, 19, 54–58). CD40-induced IL-12 release subsequently plays a role in Th1 polarization, thereby influencing the nature of the immune response. In addition, CD40 activated DC can directly induce CD8$^+$ CTL responses (59, 60). Hence, B cell and APC activation by CD40-CD40L interactions is believed to play a central role in both humoral and cellular immune responses. However, it should be noted that CD40-CD40L interactions are not essential in all antigenic systems (61–64), and independence from this pathway may be related to the strength and persistence of the antigenic stimulus and/or redundancy in costimulatory pathways (65). In addition, stimulation of vascular EC through CD40 induces the expression of the adhesion molecules CD62E, VCAM, and ICAM, which facilitate leukocyte infiltration into sites of inflammation (16–19). Finally, activated platelets express CD40L, and therefore can stimulate EC through CD40 triggering an inflammatory response (66). Hence, the CD40-CD40L pathway may serve to link vascular injury or trauma to the adaptive immune response (reviewed in Ref. 13).

Given the significance of the CD40-CD40L pathway in many diverse aspects of the adaptive and innate defense systems, it is readily apparent that these molecules may act at multiple levels in the transplant rejection process. This study focused on the role of donor and recipient CD40 expression, as opposed to the role of CD40L, which has received the majority of attention in transplantation (reviewed in Ref. 13). The critical observations of this study include the findings that BALB/c mice rejected cardiac allografts in a CD40-independent fashion that was reflected by induction of a Th1 response (Figs. 1 and 2). In contrast, allograft rejection by C57BL/6 mice was dependent on CD40, and CD40 played a critical role in Th1 priming in these mice (Figs. 3 and 4). In fact, CD40 expression by the graft was essential for optimal Th1 priming in these mice, in that WT C57BL/6 recipients of CD40$^{-/-}$ BALB/c allografts exhibited muted Th1 responses relative to their counterpart recipients of WT grafts. This effect of graft CD40 did not appear to be at the level of effector T cell recruitment into the graft, as these CD40$^{-/-}$ allografts were rejected by WT recipients, albeit in a slightly delayed fashion. It is possible that this effect on T cell priming may be at the level of activation of the allograft's "passenger" APC. In this model, donor DC migrate out of the graft and migrate to the recipient's spleen, where they stimulate graft-reactive T cells (67). As

CD40 plays a critical role in APC activation, IL-12 production, and maturation into competent APC that facilitate Th1 development, it is possible that allograft-associated $CD40^{-/-}$ DC are inefficient at stimulating Th1 priming in the recipient's spleen.

Nonetheless, recipient $CD40^{-/-}$ expression was essential for allograft rejection in C57BL/6 mice. $CD40^{-/-}$ C57BL/6 mice failed to reject either WT or $CD40^{-/-}$ allografts, indicating that CD40 expression by the graft was insufficient to over-ride CD40 deficiency in the recipient. This indicates that effector cell development is dependent upon CD40 expression, either by the effector cell itself or by the recipient APC. Macrophages, like DC, are activated through CD40 to produce proinflammatory mediators (reviewed in Refs. 9 and 19). If graft rejection is mediated by activated macrophages and a delayed-type hypersensitivity response (1, 2), CD40 may be required for macrophage effector function in delayed-type hypersensitivity. It should be stressed that there are multiple potential effector mechanisms of graft rejection, and these diverse effector mechanisms are called into play under distinct environmental conditions (reviewed in Refs. 43 and 68).

Our finding that allograft rejection by C57BL/6 mice is dependent upon CD40 is of interest because the original report documenting that costimulatory requirements were mouse strain dependent indicated that C57BL/6 mice were less susceptible to treatment with anti-CD40L mAb and CTLA4Ig (26). However, several differences between the study by Williams et al. (26) and our current report may shed light on the perceived discrepancy in the experimental findings. First, Williams et al. (26) evaluated the response to skin allografts, which are rejected in mice that are depleted of $CD4^+$ cells. Unlike skin allografts, rejection of cardiac allografts is dependent upon $CD4^+$ cells (69, 70). Indeed, $CD4^+$ and $CD8^+$ cells have differential costimulatory requirements, with $CD8^+$ cells generally being less susceptible to therapies that target CD40L (reviewed in Ref. 71). In addition, these two studies used distinct approaches to disrupting the CD40-CD40L pathway. Williams et al. (26) used anti-CD40L mAb, whereas the current study evaluated the role of CD40 in the rejection response. As emphasized throughout this discussion, targeting CD40 as opposed to CD40L is likely to yield distinct immunologic outcomes.

Why is CD40 not required for BALB/c mice to mount Th1 responses and reject C75BL/6 cardiac allografts? The answer to this is not readily apparent and may be multifactorial. For example, passenger APC in C57BL/6 allografts may be qualitatively or quantitatively more immunostimulatory than those APC that migrate out of BALB/c cardiac allografts and therefore may be more capable of stimulating graft-reactive T cells in a CD40-independent fashion. Indeed, when $CD40^{-/-}$ BALB/c DC were injected i.v. into $CD40^{-/-}$ C57BL/6 recipients, T cell priming was induced (Fig. 7), and the $CD40^{-/-}$ allografts were rejected (Fig. 6). This is in keeping with the idea that the strength and persistence of the antigenic stimulus may influence the requirement for CD40-CD40L interactions, as discussed above (61–64). The number, migration patterns, and persistence of these transplant-associated APC are currently under investigation.

It should be noted that the propagation of bone marrow-derived DC induces some degree of maturation (34), and the $CD40^{-/-}$ DC used in this study expressed detectable levels of CD80 and CD86 (data not shown). Hence, it is possible that culture-induced CD80/CD86 expression by DC, which is normally up-regulated on DC through CD40 stimulation (reviewed in Ref. 9), may play a role in the ability of donor-derived $CD40^{-/-}$ DC to stimulate allograft rejection (Fig. 6) and donor-reactive T cell responses (Fig. 7). However, syngeneic $CD40^{-/-}$ DC, which also express CD80 and CD86, failed to induce allograft rejection. Further, WT third-party DC induced significantly delayed allograft rejection and muted donor-reactive T cell responses relative to their donor-derived $CD40^{-/-}$ counterparts. These observations suggest that DC expression of CD80 and CD86 is not the only contributing factor to stimulating rejection, and expression of the appropriate set of Ags by DC is critical.

Despite the fact that CD40 was not required for allograft rejection in $CD40^{-/-}$ BALB/c recipients, anti-CD40L therapy proved to be markedly protective when WT BALB/c were transplanted with WT C57BL/6 cardiac allografts (allograft survival, >60 days; $n = 5$). These observations further indicate that anti-CD40L therapy does not simply block CD40-CD40L interactions and support a wider range of activities for anti-CD40L Abs (reviewed in Ref. 13), which may include complement-mediated lysis (41), FcR-mediated removal of CD40L-expressing T cells (42), and/or T cell apoptosis (12). CD40-CD40L interactions clearly regulate multiple aspects of both the innate and adaptive defense systems. Hence, a better understanding of the individual contributions of these molecules to the rejection process should provide insight for the development of therapies aimed at targeting the CD40-CD40L costimulatory pathway after transplantation.

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