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*J Immunol* 2000; 165:3506-3518; doi: 10.4049/jimmunol.165.6.3506

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Host CD40 Ligand Deficiency Induces Long-Term Allograft Survival and Donor-Specific Tolerance in Mouse Cardiac Transplantation But Does Not Prevent Graft Arteriosclerosis

Koichi Shimizu,* Uwe Schönbeck,* François Mach,* Peter Libby,* and Richard N. Mitchell††

Although interruption of CD40-CD40L interactions via their respective mAbs yields prolonged allograft survival, the relative importance of CD40 or CD40L on donor or host cells remains unknown. Moreover, it is uncertain whether any allospecific tolerance occurring with CD40-CD40L blockade will also prevent allograft arteriopathy, the major long-term limitation to transplantation. Therefore, we performed cardiac transplantations using CD40L-deficient (CD40L−/−) mice to investigate the mechanisms underlying prolonged allograft survival. Without immunosuppression, wild-type (WT) hosts rejected allo-mismatched WT or CD40L−/− heart allografts within 2 wk. Conversely, allografts in CD40L−/− hosts beat vigorously for 12 wk. Anti-CD40 treatment did not induce graft failure in CD40L−/− recipients. Although graft-infiltrating cells were reduced ~50% in CD40L−/− hosts, the relative percentages of macrophages and T cell subsets were comparable to WT. IFN-γ, TNF-α, and IL-10 were diminished commensurate with the reduced cellular infiltrate; IL-4 was not detected. CD40L−/− recipients did not develop IgG alloantibodies and showed diminished B7 and CD28 expression on subsets of graft-infiltrating cells. CD40L−/− transplant recipients developed allospecific tolerance to the donor haplotype; second set donor skin grafts engrafted well, whereas third-party skin grafts were vigorously rejected. By MLR, splenocytes from CD40L−/− allograft recipients also demonstrated allo-specific hyporesponsiveness. Nevertheless, allografts in CD40L−/− hosts developed significant graft arteriosclerosis by 8–12 wk posttransplant. Therefore, we propose that early alloresponses, without CD40-CD40L costimulation, induce allospecific tolerance but may trigger allo-independent mechanisms that ultimately result in graft vasculopathy. The Journal of Immunology, 2000, 165: 3506–3518.
were obtained from Taconic Farms (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME). B6 and B/c WT mice, at least 10th generation backcrossed on the B6 background, were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 and B/c WT mice were obtained from Taconic Farms (Germantown, NY) or The Jackson Laboratory. C3H/HeJ (C3H, H-2b) mice were obtained from The Jackson Laboratory. The mice were maintained in the Harvard Medical School animal facilities on acidified water. Sentinel animals in the same room were surveyed serologically and were consistently negative for viral pathogens. All experiments conformed to approved animal care protocols.

**Vascularized heterotopic cardiac transplantation**

Allografts were vascularly anastomosed in an intraabdominal location using the technique described by Corry et al. (12), as modified by Nagano et al. (13). Graft ischemic time was typically 20–25 min and total operative time was 45–50 min with a success rate (beating hearts) of more than 90%. All grafts were evaluated daily by measuring the force of palpable heart beat, adopting a scoring system with a scale of 0–4 as described by Corry et al. (12). Rejection of heart grafts was not associated with death of recipients.

**Skin transplantation**

Full-thickness tail skin grafts (~1 cm²) were transplanted onto the thorax of recipient mice, stitched with 4-0 Ethibond (Johnson & Johnson, Somerville, NJ) for 7 days. Rejection was defined as the complete loss of viable graft tissue.

**T cell proliferation assays**

One-way MLR and immobilized anti-CD3 Ab-induced T cell proliferation assays were performed using whole naive spleenocyte populations of WT and CD40L−/− B6 or B/c primed spleenocytes harvested from WT or CD40L−/− B6 recipients 4 wk following transplant. Fifty microliters (5.0 × 10⁶ cells) each of responder B6 spleenocytes and irradiated (30 Gy) stimulator B/c or C3H/HeJ (C3H, H-2b) spleenocytes were added in quadruplicate to 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA). Alternatively, 5.0 × 10⁶ responder B6 spleenocytes were cultured in 96-well microtiter plates precoated with anti-CD3 mAb (1 μg/ml). Cells were cultured at 37°C under 5% CO₂ atmosphere for 1–5 days; proliferation at each time point was assessed by pulsing the wells for 6 h with 1 μCi of tritiated thymidine (New England Nuclear, Boston, MA). Proliferation was measured as incorporated radioactivity (cpm) using a Betaplate scintillation counter (LKA Pharmacia). Results were expressed as the mean ± SEM.

**Histological evaluation**

For assessment of parenchymal rejection (PR) and GAD, grafts were analyzed in a blinded fashion using sections stained by hematoxylin and eosin, and elastic tissue stains, as described previously (13). The severity of PR was graded using a scale modified from the International Society for Heart and Lung Transplantation (0, no rejection; 1, focal mononuclear cell infiltrates without necrosis; 2, focal mononuclear cell infiltrates with necrosis; 3, multifocal infiltrates with necrosis; 4, widespread infiltrates with hemorrhage and/or vasculitis) (13, 15). The overall GAD score was averaged from the scores of all epicardial and intramyocardial arteries and arterioles in each graft (0, vascular stenosis <10%; 1, 10–25% stenosis; 2, 25–50% stenosis; 3, 50–75% stenosis; 4, >75% stenosis), as described previously (13). Typically, 10 or more vessels were graded in each graft; scores were expressed as the mean ± SD.

**Extraction of lymphocytes from spleen and cardiac allograft**

Spleens were removed and passed through a cytospin into RPMI 1640 (Life Technologies, Grand Island, NY). The cells and residue were pelleted at 1200 rpm for 5 min, and resuspended in 5 ml Tris-aminonan chloride buffer (0.83% NH₄Cl, 5 mM Tris buffer, pH 7.2) at 37°C for 5 min to lyse RBC. Lymphocytes were washed twice more in PBS, and resuspended in RPMI with 10% FCS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 57 μM 2-ME (C/10 medium) at a concentration of 1 × 10⁶ cells/ml.

Sections from harvested cardiac allografts were minced with a sterile blade and incubated in 10 ml buffered saline with 2% BSA and 2 mg/ml collagenase at 37°C for 2 h. The cells were strained through a 70 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Lymphocytes were isolated from these cells by Ficol (Organon Teknika, Durham, NC) density gradient centrifugation for 20 min at 800 rpm. After washing twice in RPMI 1640, lymphocytes were resuspended in C/10.

**Antibodies**

All of the Abs used for T cell proliferation assay, cytokine ELISA, and flow cytometry analysis were purchased from PharMingen (San Diego, CA). For T cell proliferation assay, anti-CD3 mAbs (Hamster IgG, clone 145-2C11) were used. For intracellular cytokine staining, extracted cells were stained with either a biotinylated rat IgG1 (R3-34) as isotype-matched control or biotinylated anti-IFN-γ (XMG1.2) or biotinylated anti-TNF-α (MP6-XT). For CD28, CTLA-4, or CD80 staining, extracted cells were stained with either a PE-conjugated hamster Ig (anti-TNF; 2.4.6-trinitrophenol) as isotype matched control or anti-CD28-PE (37.31), anti-CTLA-4-PE (9H10), or anti-CD80-PE (16-10A1). For CD40 and CD86 staining, extracted cells were stained with either a PE-conjugated rat IgG2a (R35-95) as isotype-matched control or anti-CD40-PE (3/23) or anti-CD86-PE (GL1). For CD4, CD8, or CD11b staining, anti-CD4-PerCP (RM4–5), anti-CD8-PE (53-6.7) or biotinylated-anti-CD8 (53-6.7) and streptavidin-APC as second Ab, and anti-CD11b-FITC (M1/70) were used. For ELISA, purified or biotinylated anti-IFN-γ (rat IgG1, clone XMG1.2), or anti-TNF-α (rat IgG1, clone MP6-XT3) were used.

For Ig ELISA or measurement of serum alloantibodies using flow cytometry, purified anti-IG-H (H + L) or IgM, alkaline phosphatase-conjugated anti-IgG (H + L) or IgM Abs, or FITC-conjugated anti-IgG (H + L) or IgM Abs were used (Southern Biotechnology Associates, Birmingham, AL). For CD40 stimulatory mAb treatment experiment, CD40L−/− cardiac transplant recipients were treated with anti-CD40 mAbs (rat IgG2a, clone 3/23), which was a generous gift of Drs. Alex Macadam and Arlene Sharpe (Brigham and Women’s Hospital, Boston, MA).

**Cytokine ELISA**

Sandwich ELISA was performed using paired Abs (anti-IFN-γ and biotinylated anti-IFN-γ mAbs (PharMingen), and streptavidin-HRP). Briefly, 50 μl of purified anti-cytokine Abs (2 μg/ml) were coated overnight on 96-well microtiter plates (Becton Dickinson, Mountain View, CA) at 4°C. After blocking the plates with 2% BSA in PBS at room temperature for 1 h, 50 μl of the sample supernatant (diluted 1:2) from MLR culture was added followed by incubation for 6 h at room temperature. The plates were washed with PBS containing 0.01% Triton-X and then reacted with 50 μl of biotinylated anti-cytokine Abs (1 μg/ml) for 45 min. After washing six times with PBS containing 0.01% Triton X, plates were incubated with peroxidase-labeled streptavidin for 30 min, followed by addition of 2,2'-azino bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO) for colorimetric reaction. Absorbance was measured using a microplate reader Enmax (Molecular Devices Corporation, Sunnyvale, CA). Standard curves for each cytokine were generated using recombinant cytokines (RIFNg; Biosource International, Camarillo, CA).
Ig ELISA

Sandwich ELISA was performed using paired Abs (purified anti-IgG (H+L) or IgM, and alkaline phosphatase-conjugated anti-IgG (H+L) or IgM mAbs. Briefly, 100 μl of purified anti-Ig Abs (5 μg/ml) were coated overnight on 96-well microtiter plates (Becton Dickinson) at 4°C. After blocking the plates with 2% BSA in PBS at room temperature for 1 h, 100 μl of the sample serum (serially diluted 1:3) were added followed by incubation for 2 h at room temperature. The plates were washed with PBS containing 0.01% Triton X and then incubated with 100 μl of alkaline phosphatase-conjugated anti-Ig Abs (1 μg/ml) for 90 min. After washing six times with PBS containing 0.01% Triton X, plates were incubated with developing reagent, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma), for 30 min. Absorbance data (at 405 nm) were collected using microplate reader Emax (Molecular Devices).

Cell surface immunofluorescent staining

Cell surface staining and flow cytometry were performed using the methods described by Stinn et al. (14). Briefly, extracted cells were incubated with FITC, PE, or PerCP conjugated surface marker Abs, anti-CD4, CD8 (2 μg/ml), or CD11b Abs (1 μg/ml) or PE conjugated costimulatory molecules Abs, anti-CD80, CD86, CD28, or CTLA4 Abs (2 μg/ml) for 20 min at 4°C. After staining, cells were washed twice with PBS and followed by washing in PBS, fixation and in 1% paraformaldehyde in PBS.

Intracellular cytokine staining

Intracellular cytokine staining and flow cytometry were performed using the methods described by Stinn et al. (14). Briefly, extracted cells were incubated with 25 μM ionomycin (Sigma), and 10 ng/ml PMA (Sigma) for 4 h at 37°C under a 5% CO₂ humidified atmosphere. Brefeldin A (10 μg/ml, Sigma) was added for the duration of the culture to block cytokine secretion and thereby improve detection. After stimulation, cells were centrifuged at 1200 rpm for 5 min, and washed in ice-cold PBS before fixing at room temperature with 4% paraformaldehyde in PBS for 10 min. For intracellular staining, cells were permeabilized with saponin/PBS buffer (0.5% saponin (Sigma), 1% BSA, 0.1% NaN₃ in PBS). After incubation with 0.25 μg/ml Fc block (PharMingen) for 5 min, cells were labeled with 10 μg/ml of a primary biotinylated anti-cytokine Ab or biotinylated isotype-matched control Ab (PharMingen) for 30 min at room temperature. After washing twice with saponin/PBS buffer, the cells were incubated with APC-conjugated streptavidin (2.0 μg/ml) for 30 min at room temperature. The cells were washed twice with saponin/PBS buffer, washed with PBS alone to seal the membranes, and stained with FITC-, PE-, or PerCP-conjugated surface marker Abs (2.5 μg/ml) for 30 min at room temperature, followed by washing in PBS.

Flow cytometric analysis

Flow cytometry was performed on a four color FACScan flow cytometer (Becton Dickinson) using CellQuest software. Dead cells and polymorphonuclear cells were excluded on the basis of light scatter characteristics. Scatter regions for infiltrating mononuclear cells were established before each collection using stimulated splenocytes. Collection of cytokine staining data from allografts was restricted to this gated region. In total, 10,000 events within the mononuclear cells scatter region were routinely analyzed.

Total RNA extraction and RPA

Total RNA was isolated from cardiac allografts with TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. The concentration of RNA was determined by ultraviolet spectroscopy at 260 nm. The RPA was performed according to the manufacturer’s recommendations using 20 μg of total RNA from each graft heart and mck-1 or mck-3b Riboquant multiprobe RPA kits (PharMingen). After electrophoresis on 6% polyacrylamide/urea gel, the patterns of radioactivity were recorded as intensity of OD (IOD) using a Molecular Dynamics PhosphorImager. The cytokine mRNA IOD was normalized to the GAPDH gene mRNA IOD using ImageQuant software.

Measurement of serum alloantibodies

Naive splenocytes (1 × 10⁶) of B/c WT animals, prepared as described above, were incubated for 30 min at room temperature with 30 μl of 1:10 diluted sera obtained from naive B6 mice (control), or WT or CD40L-/- B6 recipients of heart transplants, 4 wk after grafting. Cells were washed twice, incubated with FITC-conjugated anti-mouse IgG (H+L) or IgM (Southern Biotechnology Associates), and PE-conjugated anti-CD8 and PerCP-conjugated anti-CD4 Abs (PharMingen). Flow cytometry was performed on a three color FACScan flow cytometer (Becton Dickinson) using CellQuest software.

Statistical analysis and transplant survival analysis

Comparative analysis of cardiac graft survival was accomplished via the Kaplan-Meier cumulative survival method and survival differences between two groups were determined using the log-rank (Mantel-Cox) test. Comparisons between two groups for cytokine and Ig ELISA, MLR, T cell proliferation, and normalization of RPA data, parenchymal rejection and graft arterial disease scores, graft-infiltrating cell number, and phenotype distribution rate were accomplished by one-way ANOVA. Comparison over the time course of parenchymal rejection and graft arterial disease scores and normalization of RPA data were analyzed by ANOVA for repeated measures using StatView 4.5 for Macintosh (Abacus Concepts, Berkeley, CA). Data are expressed as mean ± SE (SEM), p < 0.05 was considered statistically significant.

Results

Cardiac allografts in CD40L-/- recipients survive long-term without immunosuppression

Vascularized heterotopic abdominal cardiac transplantations were performed using total allogeneic mismatched combinations of BALB/c (H-2b) (B/c) and WT or CD40L-/- C57BL/6J (H-2b) (B6) mice. WT B6 donor hearts were rejected by 13.1 ± 4.9 days when transplanted into WT B/c recipients (n = 11); CD40L-/- B6 donor hearts were rejected by 13.0 ± 4.6 days in WT B/c recipients (n = 6; no significant difference) (Fig. 1A). B/c hearts in B6 hosts ceased functioning by 8.4 ± 1.4 days (n = 11). In contrast,
all B/c donor hearts in CD40L−/− B6 recipients maintained good (4+ palpation) graft function for more than 12 wk (n = 8) (p < 0.0001) (Fig. 1B).

Long-term allografts in CD40L−/− recipients exhibit parenchymal rejection and develop graft arterial disease

By postoperative day 7, the histologic grade of PR in WT B/c allograft hearts was significantly greater for B6 WT than for B6 CD40L−/− recipients (Fig. 2A, and compare with Fig. 2, D and E). PR scores were 3.13 ± 0.52 and 2.17 ± 0.41 (p = 0.0028) in WT (n = 8) and CD40L−/− (n = 6) recipient allografts, respectively. The extent of PR did not significantly change in CD40L−/− recipient allografts with time (Fig. 2B, D–G) although there was a trend toward diminishing infiltrates. Because grafts in WT hosts failed within 2 wk of transplant, they did not develop the intimal hyperplastic lesions characteristic of GAD. However, the long-term grafts in CD40L−/− hosts showed progressively increasing severity and extent of GAD (Fig. 2, C and H).

CD40L−/− splenocytes proliferate normally to anti-CD3 Abs, and exhibit a strong primary MLR

We assessed the ability of CD40L−/− lymphocytes to respond to direct TCR ligation and allogeneic stimulation in primary MLR. CD40L−/− lymphocytes and WT cells stimulated by immobilized anti-CD3 mAbs displayed the same [3H]thymidine incorporation

**FIGURE 2.** Allograft histology, and PR, and GAD scores. A, The extent of PR is significantly greater in WT than in CD40L−/− recipient allografts harvested on postoperative day 7 (p < 0.005). B, PR in CD40L−/− recipient allografts harvested 1, 2, 4, 8, 12 wk after transplant. PR tended to decrease in CD40L−/− recipient allograft, although the PR score change was not statistically significant (repeated ANOVA with Schefé post hoc testing). C, GAD in CD40L−/− recipient allografts harvested 1, 2, 4, 8, 12 wk after transplant. GAD increased significantly in CD40L−/− recipient allograft (repeated ANOVA with Schefé post hoc testing). D–H, WT B6 (D) or CD40L−/− (E–H) recipient cardiac allograft harvested 7 days (D and E), 4 wk (F), or 12 wk (G and H) after transplant, were shown for hematoxylin and eosin (D–G) or elastic staining (H). D, There is multifocal mononuclear cell infiltration with myocardial necrosis, vasculitis and interstitial hemorrhage; E, There is focal mononuclear cells infiltration with myocardial necrosis. No vasculitis or interstitial hemorrhage is seen. F and G, Each shows multifocal mononuclear cells infiltration with myocardial necrosis (PR grade 3). H, Mild to moderate GAD (Grade 1–2).
(Fig. 3A), indicating that CD40L<sup>−/−</sup> T cells have normal proliferative capacity. Although WT lymphocytes had greater proliferation than CD40L<sup>−/−</sup> lymphocytes in a primary MLR, CD40L<sup>−/−</sup> lymphocytes still exhibited a strong allogeneic response with similar kinetics (Fig. 3B). We also examined the levels of IFN-γ and IL-4 expression in supernatants from anti-CD3 stimulated cultures or primary MLRs, at 1–5 days after plating. Levels of IFN-γ were comparable between CD40L<sup>−/−</sup> and WT responder cells in either anti-CD3 or MLR cultures (Figs. 3, D and E). No IL-4 was detected in any of these cultures (data not shown).

**Splenocytes from CD40L<sup>−/−</sup> heart transplant recipients are specifically tolerized to graft haplotype cells**

In MLR using in vivo primed splenocytes harvested 4 wk post-transplant from WT or CD40L<sup>−/−</sup> recipients, WT lymphocytes had significantly (p < 0.0001) higher [³H]thymidine uptake than CD40L<sup>−/−</sup> lymphocytes (Fig. 3B). IFN-γ secretion by WT responder cells was also significantly increased on day 1 through day 5 compared with CD40L<sup>−/−</sup> B6 responder cells (Fig. 3E). Again, no IL-4 was detected (data not shown).

To assess whether the mechanism of long-term allograft survival in CD40L<sup>−/−</sup> recipient allografts was due to the development of donor Ag specific tolerance, we compared the primary MLR and the B/c heart transplant-primed MLR to third party C3H stimulators. As shown in Fig. 3C, [³H]thymidine incorporation was comparable between the primary MLR to C3H and the MLR to C3H using B/c-primed responder splenocytes. These data demonstrate donor-specific hyporesponsiveness.

**Second set skin transplantation demonstrates allospecific tolerance of CD40L<sup>−/−</sup> heart allograft recipients**

To confirm the induction of donor-specific tolerance, we performed skin transplantation after heart transplantation. It has been demonstrated that CD40L blockade alone does not prevent allograft skin rejection (10). Similarly, skin allografts are rejected in naive CD40L<sup>−/−</sup> hosts (Fig. 4A). However, B6 CD40L<sup>−/−</sup> hosts

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**FIGURE 3.** A. Anti-CD3 Abs-induced T cell proliferation assays using naive WT or CD40L<sup>−/−</sup> B6 splenocytes. B. Primary and B/c-primed MLR using irradiated B/c splenocytes as stimulators. C. Primary and B/c-primed MLR using irradiated C3H splenocytes as stimulators. Whole spleen cells (5 × 10<sup>6</sup>/well) from WT or CD40L<sup>−/−</sup> B6 mice were stimulated with irradiated (30 Gy) stimulator cells (5 × 10<sup>5</sup>/well) or immobilized anti-CD3 mAb (A), B/c spleen cells (B), or C3H spleen cells (C) for 5 days. In B/c primed MLR, the spleen was harvested from WT or CD40L<sup>−/−</sup> B6 recipients of B/c heart allograft 4 wk after transplant. In immobilized anti-CD3 Ab-induced T cell proliferation assays, naive WT and CD40L<sup>−/−</sup> B6 responder cells showed comparable [³H]thymidine uptake (A). In primary MLR, the peak [³H]thymidine incorporation was ~2-fold higher for WT compared with CD40L<sup>−/−</sup> B6 responder cells. However, B/c primed MLR of WT B6 recipients showed ~6-fold higher [³H]thymidine incorporation compared with CD40L<sup>−/−</sup> B6 responder cells (B). In contrast, primary and B/c primed MLR of CD40L<sup>−/−</sup> B6 responder cells to third party C3H stimulator showed the same extent of [³H]thymidine incorporation (C). We also examined the levels of IFN-γ production in supernatants from anti-CD3 stimulated cultures or primary MLRs, at 1–5 days after plating. Levels of IFN-γ were comparable between CD40L<sup>−/−</sup> and WT responder cells in either anti-CD3 or MLR cultures (D and E, left graph). In contrast, IFN-γ secretion by CD40L<sup>−/−</sup> B6 responder cells was significantly decreased on day 1 through day 5 compared with WT B6 responder cells following in vivo priming (E).
with long-term surviving B/c cardiac allografts retained B/c skin grafts for more than 4 wk (Fig. 4, B and C). The third party (C3H) skin grafts were rejected within 2 wk of transplant (Fig. 4D), demonstrating the induction of allospecific tolerance.

**CD40 ligation with stimulatory Ab does not alter allograft survival in CD40L−/− recipients**

The long-term survival of allografts in CD40L−/− recipients could be due to lack of signaling via CD40 rather than via CD40L. Thus, to test whether ligation of allograft CD40 may induce allograft rejection even in CD40L−/− recipients, we performed the following study. B/c to CD40L−/− B6 cardiac transplant recipients were divided into two groups; group 1 (n = 3) received intraperitoneal injection of 100 μg/day of activating anti-CD40 Ab (3/23) for 7 days beginning at day 0 (operative day), following a protocol previously shown to be stimulatory (16). Group 2 (n = 5) received i.p. injection of 100 μg/day of isotype matched rat IgG2a for 7 days beginning at day 0 (operative day). Efficacy of anti-CD40 Abs treatment was confirmed by an increased serum IgG on postoperative day 10 (Fig. 5). Hearts functioned well in both groups with comparable beat strength at 4 wk. All donor hearts were harvested on postoperative week 4, and intracellular cytokine staining and histological studies were performed. There was no significant difference in the levels of PR and GAD score between the two groups (Table I). The apparent lower GAD score from this set of animals at 4 wk, relative to Fig. 2C is not statistically significant. The results suggest that ligation of CD40 expressed on cells of the donor heart does not play an important role in allograft rejection.

**Relative distributions of graft-infiltrating cells is not altered in CD40L−/− recipients**

To test whether long-term allograft survival in CD40L−/− recipients corresponded to differences in inflammatory infiltrates, we stained cells extracted from grafts with anti-CD4, CD8, CD11b, NK1.1, and B220 mAbs (14), and analyzed them by flow cytometry. Corresponding to the decreased PR grade, the total number of the infiltrating mononuclear cells was less in allografts harvested 6 days after transplant from CD40L−/− recipients vs WT recipients (0.8 × 10⁶ and 1.5 × 10⁶ cells/graft, respectively; p < 0.005). Nevertheless, the relative distributions of CD4+, CD8+ and CD11b+ cells were comparable (Table II). NK1.1+ cells and B220+ B cells represented <1% in both CD40L−/− and WT recipient allografts (data not shown).

**Table I. CD40-activating Ab 3/23 does not affect PR or GAD scores in allografts in CD40L−/− recipients**

<table>
<thead>
<tr>
<th>Score</th>
<th>Group 1 (3/23)</th>
<th>Group 2 (Control IgG)</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n = 3)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>1.7 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>0.6056</td>
</tr>
<tr>
<td>GAD</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.6985</td>
</tr>
</tbody>
</table>

*Score of parenchymal rejection (PR) and graft arterial disease (GAD) in CD40L−/− allograft recipients treated with CD40-stimulatory Ab (3/23) or isotype-matched control Ab. The extent of PR and GAD was comparable in both groups harvested 4 wk posttransplant. Data are represented as mean ± SEM.
Intragraft cytokines are reduced in CD40L−/− recipients, but the pattern of cytokine expression is not qualitatively different from WT hosts.

To test whether CD40L deficiency affects cytokine profiles in the allograft hearts, cytokine mRNA expression at postoperative day 6 was analyzed by RPA. Total RNA (20 μg) from each grafted heart was hybridized with 32P-labeled cytokine mRNA probes (Fig. 6, A and C). RPA data were normalized to the GAPDH gene mRNA to permit quantitation of the various mRNAs (Fig. 6, B and D). There was no detectable IL-4 or IL-5 mRNA in either CD40L−/− or WT recipient allografts (Fig. 6A). There was significantly lowered expression of IL-2 and IL-10 mRNA in CD40L−/− vs WT recipient allografts, whereas there was no significant difference in IL-15 mRNA expression. IL-6 mRNA expression was also reduced significantly in CD40L−/− recipient allografts (p < 0.0001). The largest difference between CD40L−/− and WT recipient allografts

Table II. Number and phenotype of graft-infiltrating cells

<table>
<thead>
<tr>
<th>B/c → WT B6</th>
<th>B/c → CD40L-KO B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell no./graft</td>
<td>1.53 × 10⁶</td>
</tr>
<tr>
<td>CD4+ (%)</td>
<td>33.3 ± 4.3</td>
</tr>
<tr>
<td>CD8+ (%)</td>
<td>32.7 ± 3.7</td>
</tr>
<tr>
<td>CD11b+ (%)</td>
<td>35.5 ± 3.5</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>1.01 ± 0.12</td>
</tr>
</tbody>
</table>

*p = 0.005

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**FIGURE 6.** Cytokine mRNA expression in graft hearts detected by RPA. Cardiac allografts were harvested on postoperative day 6 from B/c to WT or B/c to CD40L−/− B6 recipient mice, and the intragraft expression of the cytokine mRNA was determined by RPA. Total RNA (20 μg) from each grafted heart was hybridized with 32P-labeled cytokine mRNA template set, mCK1 (A) and mCK3b (C), purchased from PharMingen. Annealed materials were digested with RNase A and T1. Protected fragments were analyzed by electrophoresis on 6% polyacrylamide/urea gel. B and D, RPA data were normalized to the GAPDH mRNA. Bar graphs show the normalized expression of cytokine mRNA for B: IFN-γ, IL-2, IL-6, and IL-10 and for D: TNF-α, TNF-β, and TGF-β1. Data represent mean ± SEM. MIF, Macrophage-inhibitory factor; LTB, lymphotoxin B.
was in the expression of IFN-γ (*p < 0.0001*). IFN-γ mRNA was strongly expressed in WT recipient allografts, whereas being significantly reduced in CD40L−/− recipient allografts. TNF-β expression was significantly reduced in CD40L−/− relative to WT recipient allografts (Fig. 6, *C* and *D*) (*p < 0.05*). Although there was no significant difference in TGF-β2 mRNA expression between CD40L−/− and WT recipient allografts, TGF-β1 mRNA expression was significantly reduced in CD40L−/− hosts (*p < 0.0001*). Likewise, TNF-α mRNA expression was significantly reduced in CD40L−/− and WT recipient allografts (*p < 0.0001*). Notably, although some cytokines were reduced in CD40L−/− recipients relative to WT hosts commensurate with decreased cell infiltrate, the overall cytokine profiles were not qualitatively different between the two types of recipients.

To examine the time course of cytokine expression in CD40L−/− recipient allografts, RPA was performed on allografts harvested 4, 8, and 12 wk after transplant (Fig. 7; *n* = 4 for each time point). All cytokines showed a progressive and significant decrease with time (repeated ANOVA); no IL-4 or IL-5 mRNA was seen at any time point.

The cellular sources of intragraft cytokines are not changed in CD40L−/− recipients

To determine the cell source of IFN-γ and TNF-α, graft-infiltrating cells harvested on postoperative day 6 were analyzed by intracellular cytokine staining. Three color staining, using anti-CD4, anti-CD8, and anti-CD11b, was used to gate on the three populations previously defined. The fourth color was then used to analyze the cytokines (IFN-γ or TNF-α). IFN-γ and TNF-α expression by both CD4+ and CD8+ T cells was significantly reduced in CD40L−/− recipient allografts compared with WT recipient allografts (Fig. 8). TNF-α expression by graft-infiltrating CD11b+ cells was also diminished in CD40L−/− recipients compared with

![Figure 7](http://www.jimmunol.org/)
WT recipients. No IL-4 expression was detected in either CD40L−/− or WT recipient allografts (data not shown).

CD40L depletion modulates CD40, CD80 and CD86 expression on CD4+ , CD8+ , and CD11b+ graft-infiltrating cells, as well as CD28 expression on CD8+ T cells

Cardiac allografts contained numerous infiltrating mononuclear cells, including APCs such as monocytes, macrophages, and B cells, all of which express costimulating molecules such as CD80 (B7-1), CD86 (B7-2), and CD40. We hypothesized that a lack of CD40-CD40L interaction might prevent generation of allograft immunity by failing to increase B7 expression and/or by modulating the expression of CTLA-4 or CD28.

In vitro culture of B/c splenocytes with CD40L−/− or WT B6 stimulators resulted in comparable expression of CD80, CD86, CD40, and CTLA-4 (data not shown). Flow cytometry for the same costimulating molecules was also performed on infiltrating cells from B/c to WT or B/c to CD40L−/− recipient allografts harvested on postoperative day 6. CD40, CD80, and CD86 levels on CD11b+ mononuclear cells were all lower in CD40L−/− recipient allografts than in WT recipient allografts (Fig. 9A). Interestingly, CD40, CD80, and CD86 levels on CD4+ cells and CD8+ cells were all slightly higher or unchanged in CD40L−/− recipient allografts relative to WT recipient allografts (Fig. 9A). The surface expression of CTLA-4 on both CD4+ and CD8+ T cells was comparable in both WT and CD40L−/− recipient allografts (Fig. 9B). CD28 expression on graft-infiltrating CD8+ cells was higher for WT recipients than for CD40L−/− recipient allografts, although CD28 expression from CD4+ cells was comparable (Fig. 9B).

CD40L−/− recipients have normal IgM but do not develop IgG alloantibodies

Due to the absence of CD40L, CD40L−/− mice do not class-switch their Ab isotypes and have elevated IgM levels. To test whether the isotype of recipient alloantibody correlated with long-term graft survival in CD40L−/− hosts, we incubated B/c splenocytes with serum harvested from B6 WT or B6 CD40L−/− recipients of B/c hearts. Bound alloantibodies were then detected by FITC-conjugated anti-mouse IgG or IgM and flow cytometry. As shown in Fig. 10, by 4-wk posttransplant, IgG Abs reactive with donor CD4+ T cells (and CD8+ T cells, not shown) were present in WT allograft recipients but were weakly expressed, if at all, in CD40L−/− hosts. IgM alloantibodies were comparably generated in both WT and CD40L−/− recipients. Staining for Ig in
CD40L^−/− hosts 4-wk posttransplantation was negligible (not shown), indicating that the circulating Abs detected by flow cytometry are not being affected by any intragraft accumulation.

## Discussion

The studies presented here show conclusively that the absence of CD40L on recipient immune cells leads to long-term survival of complete allogeneic mismatched cardiac grafts. Moreover, allograft survival in CD40L^−/− recipients is not affected by CD40 ligation using stimulatory mAbs. The CD40L^−/− recipients of these grafts develop specific tolerance to the cardiac graft alloantigens. However, allografts in CD40L^−/− recipients undergo early moderate parenchymal rejection as assessed histologically, and despite the induction of allo-specific tolerance, go on to develop graft arterial disease. We previously showed that a 4-day transient allograft rejection followed by repeated anti-CD4 and anti-CD8 treatment to eliminate any further allospecific responses still resulted in GAD 8–12 wk later (17). The current work extends that

![Figure 9](http://www.jimmunol.org/)
CD40L may induce tolerance and promote long-term cardiac allograft specific tolerance, comparable to that seen using anti-CD40L therapy. In contrast, the MLR of CD40L−/− recipient mice 4 wk after transplantation, the MLR to donor B/c allogeneic cells was significantly reduced using 3/23 (data not shown) and 3/23 induced recipient Ig class switching, the allografts were not rejected. These results indicate that CD40 ligation directly on either host or donor cells does not play a role in allograft failure.

Thus, although CD40L blockade may lead to long-term graft survival and even alloantigen specific tolerance, early low-level alloresponses may trigger vascular responses that ultimately result in graft failure.

Larsen et al. (10) showed that anti-CD40L mAb could prevent murine cardiac allograft rejection, and combinations of anti-CD40L mAb and CTLA4-Ig, IgG2a, IgG1, or IgG2b initiated at the time of transplantation were synergistic, leading to long-term survival. Although anti-CD40L mAb alone did not affect the expression of T cell cytokines or of B7 molecules, the combination of CTLA4-Ig plus anti-CD40L inhibited the expression of IL-2, IL-4, IL-10, and IFN-γ, as determined by RT-PCR. Kirk et al. (18) also demonstrated that anti-CD40L mAb treatment allowed long-term renal allograft survival in monkeys and long-term survivors lost their mixed lymphocyte reactivity in a donor-specific manner. In those experiments, the relative role of CD40 or CD40L on donor or recipient cells could not be ascertained. Here, we unambiguously demonstrate that absence of CD40L on host cells is responsible for the graft survival. That T cells from CD40L−/− and WT mice are comparably stimulated by anti-CD3 treatment indicates that CD40L depletion per se does not cause global T cell unresponsiveness.

Induction of donor-specific tolerance has become a major goal for innovation in organ transplantation. This study examined potential pathways by which host CD40L depletion could result in allograft survival and promote allospecific tolerance. In WT hosts 4 wk after transplantation, the MLR to donor B/c allogeneic cells increased significantly relative to the primary MLR before transplantation. In contrast, the MLR of CD40L−/− recipients to donor allogeneic B/c cells decreased relative to the primary MLR before transplant. The decreased responses were donor-specific because response to third-party cells (C3H) remained unchanged before and after transplantation. Similarly, second set B/c skin grafts in CD40L−/− recipients of B/c heart allografts were retained, whereas third-party (C3H) skin grafts were promptly rejected. Thus, cardiac transplantation into CD40L−/− hosts results in allospecific tolerance, comparable to that seen using anti-CD40L mAbs (10, 18).

There are two possible mechanisms by which absence of CD40L may induce tolerance and promote long-term cardiac allograft survival. First, CD40L may interact with CD40 on donor heart endothelial cells and myocytes to induce events that cause endothelial cell and myocardial dysfunction, and eventual graft failure. A second possibility is that CD40L may be a critical receptor for T cell costimulation via CD40 on APCs. In this case, depletion of T cell CD40L may result in reduced T cell activation or T cell cytokine production, and even T cell anergy.

To examine the first hypothesis, we treated CD40L−/− allograft recipients with 3/23 anti-CD40 stimulatory Abs. Although cytokine expression (IFN-γ and TNF-α) by T cells was relatively increased using 3/23 (data not shown) and 3/23 induced recipient Ig class switching, the allografts were not rejected. These results indicate that CD40 ligation directly on either host or donor cells does not play a role in allograft failure.

Instead, the present study supports the second hypothesis that long-term graft survival in CD40L−/− hosts is associated with diminished T cell activation and subsequent effector functioning. Besides overall reduced inflammatory infiltrates, IFN-γ and TNF-α mRNA expression were significantly reduced in CD40L−/− recipient allografts. In addition, TNF-β, IL-2, IL-6, IL-10, and TGF-β1 mRNA were also reduced in CD40L−/− hosts. Flow cytometry shows that the reduction in these cytokines was due to diminished production by both CD4+ and CD8+ graft-infiltrating T cells.

Recently, Hancock et al. (19) showed that prolongation of graft survival by anti-CD40L mAb was accompanied by inhibition of Th1 cytokine (IL-2 and IFN-γ) and up-regulation of Th2 cytokines (IL-4 and IL-10). They suggested that Th1 to Th2 immune deviation was a principal mechanism by which blockade of CD40-CD40L interactions induced long-term allograft survival. In experimental and clinical transplantation, rejection has often appeared to correlate with the detection of Th1 rather than Th2 cytokines (19). Such observations are the basis of the Th1/Th2 paradigm which predicts that if rejection correlates with Th1 dominant cytokines, Th2 induction may result in graft tolerance (20). This contrasts with our data, showing that blockade of CD40L reduced Th1 cytokines (IL-2 and IFN-γ) and up-regulation of Th2 cytokines (IL-4 and IL-10), and no IL-4 and IL-5 (Th2 cytokines) were detected in either WT or CD40L−/− recipient allografts. Thus, although the Th1/Th2 paradigm is attractive, the available data do not support such a clear distinction. Indeed, the acute failure of allogeneic hearts in IFN-γ−/− mice (13) or IL-4−/− mice (21) appear to rule out a strict requirement for either of the cytokines in graft rejection. In fact, IFN-γ and IL-4 may both play important roles in long-term allograft survival (22, 23).

TNF or IL-6 have been implicated as major effectors of graft failure in rejection (24–27). Elevated TNF may cause neutrophil and endothelial activation and induce the expression of adhesion molecules on endothelial cells resulting in accumulation of leukocytes in the transplanted organ (25). Coito et al. (28) demonstrated that anti-TNF Abs prevented allograft rejection in murine heart transplantation. Russell et al. (29) also demonstrated that gene transfer of TNF or IL-6 mRNA during transplantation declined significantly as compared with syngeneic grafts. In our study, elevation of TNF and IL-6 mRNA during transplantation declined significantly in CD40L−/− recipient allografts. Our flow cytometry results also demonstrated greatly reduced expression of TNF-α by CD4+ and CD8+ T cells in CD40L−/− recipients. Thus, reduced TNF and IL-6 production may be important for acute allograft survival.

Another potential mechanism by which blockade of CD40-CD40L could prevent transplant rejection is altering B7-CD28 co-stimulation pathways (10). Several reports suggest that a primary...
role of activated Th cells in the generation of CD4-dependent cell-mediated immunity was to provide CD40 ligation on APC presumably to increase B7 expression (30–32). Interactions between B7 family (CD80 and CD86) and CD28 increase TCR signals and synergize with various cytokines (IL-4, IL-6, and IL-10). Anti-CD86 mAbs can block MLR and stimulation of T cells by APCs (33) and allografts in recipient mice deficient in both B7-1 and B7-2 functioned for at least 12 wk (34).

Confirming prior work (35), we found no difference in costimulator expression in MLR with WT or CD40L−/− stimulators (data not shown). However, intragraft CD11b+ cells have lower CD80 and CD86 expression in CD40L−/− recipient allografts, relative to WT recipient allografts. Interestingly, CD80 and CD86 expression by CD4+ and CD8+ cells were either unchanged or increased in the CD40L−/− recipient allografts, compared with WT allografts. These results agree with the previously reported finding that total CD80 and CD86 transcripts in cardiac allografts were not reduced by blockade of the CD40 pathway (36). We suggest, therefore, that the reduced expression of CD80 and CD86 by CD11b+ cells in CD40L−/− recipient allografts is most relevant. Interestingly, even in the setting of reduced CD80 and CD86 expression on CD11b+ APC, cell surface expression of CTLA-4 by CD4+ and CD8+ cells was comparable in WT and CD40L−/− recipient allografts. Moreover, CD28 expression on CD4+ cells was similar in WT and CD40L−/− recipient allografts, although CD8+ cells from CD40L−/− hosts showed lower CD28 levels. Taken together, reduced B7 expression on macrophage APC may be an important pathway by which blockade of CD40-CD40L signaling induces tolerance. Consistent with this hypothesis, Tan et al. (3) demonstrated that the B7 antagonist, CTLA4-Ig blocked the human mixed lymphocyte reaction and induced Ag-specific unresponsiveness.

The present experiments also evaluated the activation status of the graft-infiltrating cells by flow cytometry at 1 wk after transplant (data not shown). Comparable levels of CD44 and CD69 (very early activation Ag) were seen in both WT and CD40L−/− recipient allografts and comparable levels of CD44 and CD69 were detected in the graft-infiltrating cells by flow cytometry at 1 wk after transplantation and induced Ag-specific unresponsiveness. Thereby, we demonstrated that the B7 antagonist, CTLA4-Ig blocked the human mixed lymphocyte reaction and induced Ag-specific unresponsiveness.

Our work found no IgG alloantibodies in CD40L−/− recipients of B/c heart allografts, suggesting that lack of Ig class-switching may be relevant to the long-term survival in CD40L−/− recipients. Nevertheless, IgG Abs are not the only explanation, because induction of IgG by 3/23 anti-CD40 mAbs did not induce allo graft failure. Moreover, despite induction of tolerance and long-term allograft survival, and in the absence of allo specific IgG, the hearts in CD40L−/− recipients still developed GAD.

Our findings support the view that CD40L on host cells plays an important role in the regulation of allogeic responses and induction of donor-specific tolerance to cardiac allografts in the mice. This outcome is potentially mediated by regulation of accessory molecule expression on APC, and is also associated with reduced cytokine expression and lack of IgG allo-specific Abs. More importantly, however, the early transient parenchymal rejection which may be required to induce allograft tolerance (42, 43), likely triggers a cascade of Ag nonspecific effectors which will culminate in chronic vascular lesions and eventually lead to ischemic allograft failure. Although combined treatment with CTLA4-Ig and anti-CD40L mAb did ameliorate GAD in an aortic allograft model (11), this may not be attributable to a unique beneficial effect of anti-CD40L treatment but rather due to complete immunologic blockade, as we have seen with chronic anti-CD4 and anti-CD8 treatment (17). Similarly, concomitant anti-CD40L mAb and donor cell administration led to long-term survival without GAD in a mouse heart transplant model; however, the relative contribution of donor cells vs CD40L blockade cannot be assessed (9). Thus, development of tolerance alone may be insufficient to insure successful indefinite graft functioning; it is also critical to address the underlying mechanisms of graft arteriosclerosis.

Acknowledgments

We thank Eugenia Shvartz, Elissa Simon-Morrissey, and Gregory Russo for their technical expertise; Karen E. Williams for her assistance with the preparation of the manuscript; and Drs. Abul K. Abbas and Cheryl London for their critical reading of the manuscript. We also thank Rodger D. Middleton and his staff at the animal facility of Brigham and Women’s Hospital and Harvard Medical School for their excellent management of experimental animals.

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