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The CD154-CD40 T Cell Costimulation Pathway Is Required for Host Sensitization of CD8\(^+\) T Cells by Skin Grafts Via Direct Antigen Presentation\(^1\)

Yuan Zhai,* Xiu-Da Shen,* Feng Gao,* Ana J. Coito,* Barbara A. Wasowska,† Alan Salama,‡ Isabela Schmitt,‡ Ronald W. Busuttil,* Mohamed H. Sayegh,‡* and Jerzy W. Kupiec-Weglinski\(^2,3\)*

Although the CD154-CD40 T cell costimulation pathway has been shown to mediate alloimmune responses in normal recipients, little is known about its role in sensitized hosts. In this work, by using novel models of cardiac allograft rejection in skin-sensitized CD154- and CD40-deficient mice, we reaffirm the key role of CD154-CD40 signaling in host sensitization to alloantigen in vivo. First, we identified CD8\(^+\) T cells as principal effectors in executing accelerated rejection in our model. Disruption of CD154-CD40 signaling in recipients at the T cell side (CD154-deficient) but not at the APC side (CD40-deficient) abrogated accelerated (<2 days) rejection and resulted in long-term (>100 days) graft survival. This suggests that the CD154-dependent mechanism in host CD8\(^+\) T cell sensitization operates via the direct Ag presentation. Then, in comparative studies of alloimmune responses in CD154-deficient and wild-type recipients, we showed that, although alloreactive B cell responses were inhibited, alloreactive T cell responses were down-regulated selectively in the CD8\(^+\) T cell compartment, leaving CD4\(^+\) T cells largely unaffected. This unique alteration in host alloreactivity, seen not only in peripheral lymphocytes but also in allograft infiltrate, may represent the key mechanism by which disruption of CD154-CD40 signaling prevents sensitization to alloantigen in vivo and leads to long-term allograft survival. *The Journal of Immunology*, 2002, 169: 1270–1276.

Deleterious sensitization to donor MHC Ags through random blood transfusions, prior failed grafts, or pregnancies is among the most critical of problems facing clinical organ transplantation in terms of magnitude and impact (1–3). Indeed, as many as 40% of patients on transplant waiting lists have elevated levels of broadly reactive alloantibodies to prospective donors. These sensitized patients are often precluded from receiving a transplant or may experience an increased rate of early rejection episodes, which are often irreversible or difficult to treat with currently used immunosuppressive agents. Our better understanding of the immune mechanisms in host sensitization to alloantigens should result in the development of much-needed novel therapeutic approaches for the management of sensitized patients.

CD154 (CD40 ligand), a member of the TNF gene family, is expressed predominantly on mature activated CD4\(^+\) T cells and other cell types, but not on resting T cells (4). The interaction between CD154 and CD40, a glycoprotein receptor on APCs, provides a costimulatory signal that is essential for development of both cellular and humoral immune responses to T-dependent Ags (5, 6). The efficacy of CD154-targeted therapy to abrogate the rejection response and to markedly prolong allograft survival in rodents and subhuman primates has been well established (7–9). This highlights the role of the CD154-CD40 activation pathway in the immune cascade leading to acute allograft rejection. However, the actual mechanism by which CD154 blockade interferes with host alloimmune responses remains to be elucidated. Recently it has been shown that, although anti-CD154 mAb treatment prevented CD4\(^+\) T cell-mediated rejection, it may have little or no effect on CD8\(^+\) T cell activation, proliferation, differentiation, and homing to the target organ (10). Apart from a single report in which CD154 mAb treatment ameliorated blood transfusion-induced sensitization to pancreatic islets (11), little is known about the role of CD154 signaling in sensitized hosts. It also remains to be determined whether the blockade of CD154 costimulatory signals in sensitized hosts affects graft rejection via CD4\(^−\) or CD8-dependent mechanism.

To investigate mechanisms of host sensitization to alloantigens in vivo, and to further address the role of CD154 in the rejection cascade, we have developed a stringent model of vascularized cardiac allograft rejection in skin-sensitized mice (BALB/c→B6/129; accelerated rejection in ≈36 h) (12). The sequence of events in this experimental system is relevant to clinical cases of rejection in patients sensitized to MHC Ags before transplantation. In this murine model, the evidence of cellular immune activation includes generation of high CTL activity in vitro and infiltration of the graft by mononuclear cells expressing IL-2, IFN-\(\gamma\), TNF, and IL-2R. The presence of antidonor Abs and extensive intragraft deposition of IgM, IgG, C3, and rapid neutrophil infiltration confirm cardinal features of humoral injury.
This study analyzes the mechanisms by which CD154–CD40 costimulation may affect host sensitization to alloantigen in vivo. First, we determined that CD8\(^+\), but not CD4\(^+\), T cells are the prime effectors in executing accelerated rejection. Then, by using CD154\(^-/-\) mice as recipients of skin and cardiac allografts, and by selectively targeting CD154–CD40 in vivo interactions in wild-type (WT) hosts, we confirmed the key role of the CD154 pathway in sensitized hosts. Indeed, although cardiac allografts were rejected in <36 h in WT skin-sensitized mice, they survived >100 days in CD154-deficient mice or WT mice treated with anti-CD154 mAb. In contrast, CD40-deficient mice with disrupted CD154–CD40 interactions selectively in the indirect allore cognition (for CD8\(^+\) T cells) had a normal kinetics of accelerated rejection. Thus, the CD154 blockade in the direct allore cognition pathway is critical in host allosensitization. The results of our studies are the first to document that CD154 blockade in sensitized hosts results in selective inhibition of alloreactive CD8\(^+\) but sparing of CD4\(^+\) T cell responses, both systemically and locally.

Materials and Methods

**Animals and grafting techniques**

WT BALB/c (B/c; H-2\(^d\)), B6/120 (B6; H-2\(^b\)), and CBA/Ca (CBA; H-2\(^k\)) male mice were used. We also used CD154\(^-/-\) and CD40\(^-/-\) mice of the B6 background (intercrossed at least 10 generations). All mice (8–12 wk old; 20–25 g) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were housed in the University of California animal facilities (Los Angeles, CA) under pathogen-free conditions. Orthotopic full-thickness skin grafts (~0.5 cm in diameter) from B/c donors were sutured bilaterally onto the flanks of prospective WT, CD154\(^-/-\), or CD40\(^-/-\) B6 recipients. These were then challenged 10 days later into the intra-abdominal location with variously anastomosed B/c cardiac grafts. Graft survival was assessed daily by palpation of ventricular activity. The day of rejection was defined as the day of cessation of heartbeat and was verified by autopsy and pathological examination.

**Ab therapy**

Rat anti-mouse CD4 (GK1.5) and CD8 (2.43; courtesy of Dr. H. Auchincloss, Massachusetts General Hospital, Boston, MA)–depleting mAb were administered at days −2, −1, and 0 in skin-sensitized WT recipients (0.2–0.4 mg per mouse i.v.). Anti-CD154 Ab (MR1), purchased from Bioexpress (West Lebanon, ME), was given in the sensitization phase, i.e., between skin and cardiac engraftment (0.25–0.5 mg per mouse every other day i.v.). Control recipients were treated with relevant doses of rat or hamster Ig.

**In vitro MLR**

Spleens and peripheral lymph nodes were removed and passed through a cytoscissor into RPMI 1640 (Life Technologies, Grand Island, NY). The cells and residue were pelleted at 1200 rpm for 5 min and then resuspended in 5 ml Tris-ammonium chloride buffer (0.83% NH\(_4\)Cl, 5 mM Tris buffer pH 7.2) at 37°C for 5 min to lyse RBC. After washing twice with RPMI 1640 medium with 1% FBS, cells were suspended in culture medium (RPMI 1640 medium supplemented with 20 mM HEPEs, 10 mM sodium pyruvate, 2 mM l-glutamine, 50 mM 2-ME, 1× MEM-nonessential amino acid solution, 1× MEM-vitamin solution, 1× antibiotic/antimycotic solution, and 10% FBS) at a concentration of 5 × 10\(^6\)/ml. A total of 100 µl of responder B6 cells were added into a U-bottom 96-well plate (Corning, Corning, NY) and were mixed with the same number of x-irradiated (2000 rad) stimulator cells of B6 (syngeneic), B/c (donor-type), or CBA (third-party) strains. Four replicates were used for each reaction combination. Con A (2 mg/ml) was used as a positive control. A total of 1 µCi of \([\text{3}^\text{H}]\text{Th}r\) was added to each well in the last 16–18 h of a 3-day culture. Labeled cells were harvested onto filter mats (Skatron Instrument, Sterling, VA) with a Skatron 12-well cell harvester. The cpm of the filter membranes were measured in scintillation liquid on a Beckman LS 6000iC (Beckman Coulter, Fullerton, CA).

In vivo MLR

In vivo MLR, responder cells (B6 strain) were labeled with CFSE (Molecular Probes, Eugene, OR) at 4 nM in PBS for 15 min at 37°C. The unconjugated CFSE was eliminated by washing the cells with FBS (20%) supplemented RPMI 1640 medium. The labeled cells were resuspended in PBS at 2.5 × 10\(^6\)/ml, and 200 µl of these cells (5 × 10\(^4\)) were injected into total-body x-irradiated (1000 rad) B/c (or B6 as negative controls) mice via tail vein. At day 3, spleens of the injected mice were harvested and stained with anti-mouse CD3e-R-PE (clone 145-2C11), CD4-biotin (clone H129.19), and streptavidin-CyChrome (BD Pharmingen, San Diego, CA). Topro 3 (1 nM) was added as viable dye. Four-color flow cytometry was performed on a FACSCalibur dual-laser cytometer (BD Biosciences, Mountain View, CA). Topro 3-negative cells in lymphocyte gate (viable) that stained positive for CD3 and CD4 were analyzed for CFSE intensities.

**CTL effector differentiation in vivo**

RBC-free splenocytes or lymph node cells were prepared as described above. One million cells were used for Ab staining in ice-cold PBSA (PBS with 1% BSA). Cells were first incubated with 10 µg of normal rat IgG to block Fc binding sites. After washing, cells were stained with 0.5–1 µg of rat anti-mouse CD8α-FITC (clone 53-6.7), CD2L-PE-RE (clone ME-14), and CD44-CyChrome (clone IM7) (BD Pharmingen). After washing, three-color flow cytometry was performed on a FACScan cytometer (BD Biosciences). Cells in lymphocyte gate that stained positive for CD8α were analyzed for their CD62L and CD44 expression. CTL effectors were identified as the CD8\(^+\)CD62L\(^++\)CD44\(^++\) population.

**Histology and immunohistology**

H&E and immunoperoxidase staining were used for assessment of myocardial and vascular preservation and detection of inflammatory cell infiltration. IgM/IgG deposition was detected (14). Tissues were fixed in buffered formalin or embedded in Tissue Tec OCT compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C. Cryostat sections (5 µm) were fixed in acetone and endogenous peroxidase activity was blocked by incubating the slides with 0.3% H\(_2\)O\(_2\). Normal heat-inactivated rabbit serum was used for blocking. Appropriate primary rat Ab against mouse CD3\(^+\) cells (CD3-12), CD4\(^+\) cells (CT-CD4), CD8\(^+\) cells (2.43), and monocytes/macrophages (MEMA-2) were used (Hartlan Bio-products for Science, Indianapolis, IN). Primary Abs against mouse IgG and IgM, biotinylated rabbit anti-rat IgG, and streptavidin-peroxidase-conjugated complexes were obtained from Vector Laboratories (Carpinteria, CA). The control sections were performed by replacing the primary mAb with either dilution buffer or normal rat serum. The peroxidase reaction was developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO). The sections were evaluated blindly by counting labeled cells within 10 high-power fields per section. Some Abs were analyzed in a semiquantitative fashion where the relative abundance of each one was judged as follows: negative (−), little (+), moderately abundant (++), and very abundant (+++).

**Alloantibody levels**

Antidonor Abs were measured by flow cytometry. Briefly, 50-µl aliquots containing 1.5 × 10\(^5\) B/c lymph node lymphocytes were incubated for 45 min at 4°C with 50 µl of diluted sera (1/4, 1/16, 1/64). To stain for IgM and IgG, cells were washed twice and then reacted with 50 µl of PBS plus 0.2% BSA plus 0.02% sodium azide containing a mixture of FITC-conjugated goat Ab specific for the Fc portion of mouse IgG and PE-conjugated goat Ab specific for the μ-chain of mouse IgM (Jackson Immunoresearch Laboratories, West Grove, PA). After staining for 30 min at 4°C, the cells were washed, fixed in 1% formaldehyde in PBS, and analyzed by flow cytometry using FACScan (BD Biosciences). The binding of IgM and IgG alloantibodies to class I MHC Ags on B/c target cells is expressed as the mode channel fluorescence.

Abbreviations used in this paper: WT, wild type; MST, mean survival time.
CD154 IN HOST SENSITIZATION

Results

CD8+ T cells are principal effectors in cardiac allograft rejection in sensitized mice

B/c hearts were rejected in 1.4 ± 0.3 days (mean survival time (MST) ± SD) in B6 WT mice that were sensitized with B/c skin grafts at day −10 (Fig. 1). Two groups of WT recipients of skin grafts underwent a 3-day treatment with depleting anti-CD4 or anti-CD8 mAb (day −2 to day 0). Such a delayed (8 days post-skin engraftment) depletion of T cell subsets should not disrupt T cell activation by skin grafts (sensitization), yet it should prevent alternative activation pathways leading to accelerated graft rejection. Flow cytometry confirmed complete depletion of the targeted T cell subset while maintaining both the number and function of the opposite subset (data not shown). Interestingly, as shown in Fig. 1A, unlike animals treated with anti-CD8 mAb, which lost their cardiac allografts uniformly within 24 h, those given anti-CD8 mAb rejected their transplants in an acute rather than accelerated manner (5.5 ± 1 days; p < 0.001). Thus, activated CD8+ but not CD4+ T cells are the principle effectors in executing accelerated rejection of cardiac allografts in this sensitized mouse transplant model.

It is worth pointing out that prolonged treatment with depleting CD8 mAb did not result in any further delay of allograft rejection (results not shown), indicating that, although the CD8 mechanism plays a key role in accelerated rejection, the non-CD8-mediated mechanism may contribute to the acute rejection cascade.

Cardiac allografts survive long-term after disruption of CD154 costimulation in sensitized mice

A group of CD154−/− B6 mice was then challenged with B/c skin, followed 10 days later by transplantation of B/c hearts. As shown in Fig. 1B, all cardiac allografts survived >100 days in CD154−/− recipients. In contrast, skin grafts alone (no heart grafts) were rejected in 12 ± 2 days, whereas heart grafts alone (no skin grafts) survived >100 days in otherwise unmodified CD154−/− hosts (n = 4 mice per group; data not shown). To demonstrate that these findings were not confined to the murine knockout system, in which the lack of CD154 expression is complete, sustained, and specific, we then used anti-CD154 mAb treatment to block CD154-CD40 in vivo interactions in WT mice. Indeed, infusion of MR1 mAb every other day between skin (day −10) and cardiac (day 0) engraftment prevented host sensitization, as evidenced by long-term cardiac allograft acceptance in 8 of 10 WT hosts (Fig. 1B). In contrast, controls conditioned with hamster serum rejected cardiac allografts in <2 days.

Prevention of host sensitization by disruption of CD154-CD40 signaling results from inhibition of direct T cell activation by donor APCs

Both direct and indirect T cell activation can be involved in host sensitization to alloantigen. To determine which activation pathway is dominant in our model, we took advantage of the finding that CD8+ T cells were the main effectors in our model and that CD40-deficient (B6) recipients were defective only in the indirect pathway for alloreactive CD8+ T cell activation, as allogeneic APCs in WT B/c grafts have normal CD40 expression. This is in contrast to CD154-deficient recipients, defective in both direct and indirect pathways, due to the lack of CD154 expression on responding T cells. A group of CD40−/− (B6) animals was challenged with skin grafts (B/c), followed 10 days later by transplantation of donor-type hearts. Unlike CD154−/− skin-sensitized mice, which maintained their transplants long-term, all cardiac grafts were rejected promptly in CD40−/− recipients (1.5 ± 0.5 days; Fig. 1C), comparable with skin-sensitized WT hosts. Thus, CD154-CD40 signaling that is involved in the direct CD8+ T cell activation pathway by donor APCs is critical in host sensitization to alloantigens in vivo.

T cell hypersensitivity to alloantigen in vitro is restricted to the CD8+ subset from CD154−/− recipients

Spleen lymphocytes were harvested from groups of WT and CD154−/− primed recipients of cardiac allografts at 1–2 days post-transplant and analyzed for in vitro alloreactivity in MLR assay. By that time, WT hosts undergo fulminant rejection of cardiac allografts, whereas CD154-deficient mice maintain functioning transplants despite prior donor-type skin engraftment. As shown in Fig. 2A, splenocytes from CD154-deficient hosts proliferated less vigorously against alloantigens, as compared with WT controls (donor-type, p < 0.0005; third-party, p < 0.05). Addition of Con A stimulated proliferation of T cells to similar degrees in both recipient groups, indicating no general proliferation defects in splenocytes from CD154−/− recipients. FACS staining of CFSE-
labeled splenocytes in bulk cultures identified both CD4 and CD8 phenotypes in the alloresponsive T cell population. Whereas CD154−/− CD4+ T cells proliferated normally as compared with WT CD4+ T cells (16.6 vs 9.1%, Fig. 2B), the CD154−/− CD8+ T cells had much-reduced numbers of proliferating cells compared with the WT CD8+ T cells (24.8 vs 70.1%, Fig. 2B). These results indicate in vitro hyporesponsiveness of CD154−/− splenocytes after in vitro priming, and that this hyporesponsiveness was restricted to CD8+ T cells without affecting the CD4+ T cell compartment.

**CD4+ T cells from CD154−/− recipients readily proliferate in vivo**

To further examine the proliferative response of CD4+ T cells, we conducted in vivo MLRs, which provide an ideal setting for alloreactive CD4+ T cells to differentiate between deletional and non-deletional mechanisms that may contribute to hyporesponsiveness in vitro. Hence, splenocytes from groups of WT and CD154-deficient primed hosts were harvested at days 1–2 posttransplant, labeled with CFSE, and then injected i.v. into gamma-irradiated donor-type (B/c) test mice to determine their in vivo alloreactivity. The results presented in Fig. 3 are in agreement with robust proliferation of transferred spleen cells from CD154−/− hosts, similar to that of rejecting WT littermates. CD4+ T cells were the major responsive population. This response was allospecific, as no significant proliferation was detected with cells injected into gamma-irradiated syngeneic (B6) mice. These results confirm our in vitro results that alloreactive CD4+ T cells were responsive in CD154-deficient hosts.

**CD8+ T cell differentiation in vivo is defective in CD154−/− recipients**

To further examine the function of alloreactive CD8+ cells from CD154−/− recipients, splenocytes were analyzed for the generation of CD8+ CTL effector phenotype induced by alloantigens. The expression of the CD8+ CD44hiCD62Llow phenotype correlates with cytolytic activity, as previously shown (13). Fig. 4A illustrates results of a representative experiment and depicts the percentages of CD8+ CD44hiCD62Llow spleen cells in WT and CD154−/− engrafted hosts at day 2 posttransplant. Unlike in primed WT splenocytes, the CTL effector phenotype expression by CD154-deficient splenocytes was markedly diminished, and was comparable with that of naive mice (43.1, 4.7, and 2.3%, respectively). These data were consistent with results of an in vitro cytotoxicity assay (Fig. 4B), in which target cell killing by CD154−/− T cells was markedly diminished as compared with that of WT cells (12 ± 3 and 53 ± 4%, respectively, at a 50:1 E:T ratio). Collectively, these results document abrogation of CD8+ T cell differentiation and generation of CD8+ CD44hiCD62Llow phenotype CTL effector cells, consistent with defective effector T cell function in mice deficient for CD154.

**FIGURE 2.** In vitro proliferative responses of lymphocytes from WT and CD154−/− recipients of cardiac allografts (days 1–2 posttransplant). A, One-way MLR was set up with B6 splenocytes from WT rejecting or CD154-deficient hosts bearing well-functioning B/c cardiac allografts, against irradiated (2000 rad) B6 (syngeneic control), B/c (allogenic donor-type), or CBA (allogenic third-party) splenocytes. One million of B6 mouse cells were cultured with the same number of stimulator cells or 2 mg/ml Con A (four replicates per sample) for 3 days. Cells were pulsed with 1 uCi of [3 H]TdR per well and harvested 16–18 h later, and [3 H]TdR incorporation was counted. The average cpm ± SD for each sample were charted. B, In vitro proliferative responses of CFSE-labeled spleen cells. CFSE-labeled responder cells from B6 WT or CD154−/− recipients (day 2 post-cardiac transplant) were set up in bulk cultures against x-irradiated allogenic B/c splenocytes or Con A (2 μg/ml). At day 4, cells were harvested, viable CD4+ or CD8+ cells were gated, and CFSE intensities were plotted. Representative data from three different experiments are shown. Splenocytes from CD154−/− hosts remain hyporesponsive to donor and third-party alloantigen in vitro. Results are representative of four independent experiments.

**FIGURE 3.** In vivo proliferative responses of lymphocytes from WT and CD154−/− recipients of cardiac allografts (days 1–2 posttransplant). Fifty million of CFSE-labeled splenocytes were injected i.v. into irradiated (1000 rad) B/c or B6 mice. At day 3, splenocytes from test animals were prepared for a four-color FACS analysis, viable CD3+ CD4+ cells were gated, and CFSE histograms were shown. CD4+ T cells in WT and CD154-deficient splenocytes proliferate vigorously in vivo in allogeneic B/c (but not syngeneic B6) test mice. Data from one representative experiment of four are shown.
Intragraft mononuclear cell infiltration is decreased in CD154−/− recipients

We then asked how disruption of the CD154 costimulation pathway affects host cell immune responses at the graft site. The sequential analysis of cardiac allografts harvested from sensitized WT mice at 24–30 h posttransplant showed considerable edema and myocardial degeneration, contrasting with the relatively normal histological appearance observed in the cardiac grafts harvested from primed CD154−/− hosts. Fig. 5 depicts some of the key immunohistological findings in cardiac allografts from WT and CD154−/− recipients. Thus, cardiac grafts from WT hosts were characterized by elevated myocardial infiltration, consisting predominantly of CD3+ cells (Fig. 5A; 40.5 ± 4.9). In contrast, cardiac grafts from CD154-deficient hosts showed depression in the number of CD3+ cells (Fig. 5G; 17.2 ± 8.8; p < 0.02). Interestingly, unlike CD4+ cells, which remained comparable between WT (Fig. 5B; 7.5 ± 0.7) and CD154-deficient (Fig. 5H; 6.3 ± 2.1) mice, the frequency of infiltrating CD8+ cells diminished sharply in CD154−/− hosts (Fig. 5I; 26 ± 8.7) as compared with WT controls (Fig. 5C; 71.5 ± 16.2; p < 0.01). A similar cell infiltration pattern was detected in cardiac allografts harvested at 24–30 h from WT skin-sensitized mice treated with MR1 mAb (data not shown). Hence, consistent with our findings on cells in the peripheral lymphoid organs, CD154 signals are also required for the migration of CD8+ T cells to the allograft site, as their absence (in CD154−/− hosts) or blockade (in MR1 mAb-treated WT mice) prevented sequestration of CD8+ T cells in cardiac allografts in this model.

IgM and IgG antidonor Ab responses are depressed in CD154−/− recipients

Because the CD154–CD40 pathway is critical for CD4-dependent B cell reactions, we determined systemic donor-specific alloantibody responses elicited by B/c cardiac allografts in B6 mice sensitized with B/c skin grafts. Sera were harvested from groups of untreated WT and CD154-deficient recipients at days −10 (the day of engraftment), −7, −3, 0 (the day of cardiac engraftment), +1, +2, and +7. Serially diluted sera samples were then tested by flow cytometry for IgM and IgG binding to B/c lymph node target cells. In sensitized WT engrafted hosts, IgM alloantibody response peaked at day +2, i.e., around the time of accelerated rejection (Fig. 6). The IgM alloantibody response switched to strong IgG alloantibody response, which remained elevated for up to 7 posttransplant days. In contrast, in CD154−/− recipients, serum IgM and IgG alloantibody levels remained close to background levels throughout the observation period.
To define local antidonor alloantibody responses, we used immunohistochemistry to analyze endothelial IgM and IgG alloantibody deposition at the graft site. As shown in Fig. 5, cardiac allografts from WT mice showed extensive and widespread deposits of IgM (Fig. 5E; +++) and IgG (Fig. 5F; +++) in contrast, cardiac grafts from CD154-deficient hosts were characterized by diminished staining for IgM (Fig. 5K; +) and IgG (Fig. 5L; +/-). Thus, disruption of the CD154 costimulation pathway abrogates otherwise strong systemic and intragraft antidonor IgM and IgG alloantibody responses in primed recipients.

**Discussion**

Previous studies have established the importance of CD154-CD40 signaling in the generation of T cell-mediated alloimmunity, including models of acute allograft (7–9, 14) and allogeneic tumor (15, 16) rejection, as well as donor blood-induced sensitization (11). In the present report, we provide evidence that in a more stringent skin-sensitized cardiac allograft rejection model, host sensitization to alloantigen in vivo proceeds via the CD154-CD40 costimulation mechanism in the direct allorecognition pathway. The finding that CD8+ T cells were the main effectors in graft rejection provides us with an opportunity to 1) differentiate between direct vs indirect allorecognition pathways in alloreactive CD8+ T cell activation in host sensitization, and 2) analyze the impact of CD154-CD40 costimulation, particularly in alloreactive CD8 activation. Both cellular and humoral immune responses contribute to the mechanism of transplant rejection in sensitized hosts. Hence, the question arises whether the absence/disruption of the CD154 costimulation affects T cells, B cells, or their interactions in the immune cascade that otherwise culminates in fulminant rejection of cardiac grafts in sensitized WT mice.

As previously reported, the interaction between CD40 on B cells and CD154 on T cells plays a key role in B cell activation, including proliferation, the generation of a thymus-dependent Ab response, and Ig isotype switching (17). Indeed, treatment with anti-CD154 mAb diminished the response to alloantigen on B cells in vitro (18), whereas immunization with resting B cells from CD154−/− mice induced tolerance to alloantigen (19). Treatment with anti-CD154 mAb blocks both primary and secondary immune responses to T cell-dependent Ags, as well as the development of germinal centers and the generation of memory cells (20). Moreover, individuals with type IgM syndrome, an immunodeficiency due to a mutation of the CD154 gene, express diminished serum IgG, IgA, and IgE levels and are unable to mount secondary T cell-dependent responses (21). Similarly, we found diminished IgM and IgG alloantibody levels in the serum of CD154-deficient recipients of both skin and heart allografts. Moreover, intragraft deposition of both IgM and IgG was depressed in well-functioning grafts of CD154−/− hosts as compared with that in rejecting WT controls. All these suggest that depression of Ig responses and class switching represents a relevant mechanism by which disruption of the CD154 costimulation pathway prevents sensitization to alloantigen in vivo.

As to the T cell responses, the results of our study demonstrate that splenic T cells from CD154-deficient hosts were hyporesponsive to alloantigen in vitro MLR, as compared with WT controls, consistent with a previous report by Shimizu et al. (22). However, subset analysis of the proliferating cells has revealed that the overall hyporesponsiveness in CD154-deficient hosts resulted from selective depression of CD154-deficient CD8+ T cells, whereas proliferation of CD154-deficient CD4+ T cells was virtually unaffected. In addition, CFSE-labeled CD4+ splenocytes from CD154−/− recipients proliferated vigorously in vivo following infusion into x-irradiated allogeneic test mice, similar to cells from WT controls. This indicates that alloreactive CD4+ T cells in the splenocyte pool were unaffected in our CD154−/− transplant model, at least in their proliferative responses and cytokine production (IFN-γ; data not shown) against alloantigens. In contrast, CD8+ T cells were both hyporesponsive in vitro and defective in their ability to become activated in vivo (differentiation into CTL effectors) by allograft stimulation. This defect was alloantigen specific, as polyclonal stimulation, such as with Con A or PMA/ionomycin, readily stimulated CD154−/−CD8+ T cells to proliferate and secrete cytokines (IFN-γ; data not shown) to the same extent as WT CD8+ T cells. To best of our knowledge, this is the first direct evidence that in an allograft model alloreactive CD8+ but not CD4+ T cells were severely hampered by the CD154 blockade. It is worth pointing out that, although CD154-deficient CD4+ T cells were able to proliferate, their functions may still be interrupted, particularly in the aspect of providing help for CD8+ T cells and B cells.

The mechanism by which disruption of the CD154-CD40 pathway suppresses cell-mediated immunity varies depending on the experimental model system. Although it is well established in nominal Ag model systems that CD4-dependent CD8+ T cell activation requires CD154-CD40 costimulation via indirectly activated dendritic cells by CD154-positive CD4+ T cells (23, 24), its role in alloreactive CD8+ T cells remains controversial. Alloreactive CD8+ T cells are present in high precursor frequencies and may become activated through both CD4-dependent and CD4-independent pathways. Unlike CD4-dependent CD8 activation, the requirement for CD154 costimulation in CD4-independent CD8+ T cell activation remains largely unknown. The existing literature favors the hypothesis that CD8+ alloreactive T cells, as a whole population, are less sensitive or resistant to the CD154 blockade, as shown by Jones (10), Guo (25), and Honey (26) in both major and minor MHC-mismatched allograft models. However, reports from allogeneic tumor models suggest that CD8+ T cell activation may depend on the CD154-CD40 pathway, and that activation of CD8+ T cells may be in fact CD4 independent (15, 16). In addition, it has been shown that long-term expansion of adoptively
transferred 2C TCR-transgenic CD8+ T cells in Ag-bearing F1 hosts depends on CD154 costimulation signals via CD4+ T cells (27). Our results document that disruption of CD154 signaling was indeed inhibitory to alloreactive CD8+ T cells in sensitized transplant recipients, and our preliminary data suggest that both CD4-dependent and CD4-independent activation of alloreactive CD8+ T cells may be affected in this model (Y. Zhai, manuscript in preparation).

The sparing of at least some of alloreactive CD4+ T cell function (proliferation and cytokine production) by CD154 blockade is of interest. In contrast to cells lacking CD28 signaling, which were unable to proliferate in both CD4 and CD8 compartments in vitro and in vivo (data not shown), CD154-deficient CD4+ T cells or WT CD4+ T cells under MR1 treatment all underwent vigorous proliferation after allostimulation. This may be important for long-term cardiac allograft survival after transient CD154 blockade in our model, because CD28-deficient recipients promptly lost their transplants in <2 days, whereas treatment with CTLA4-Ig only marginally prolonged cardiac allograft survival in skin-sensitized WT mice (data not shown). A similar observation (effects of CD154 vs CD28 blockade) was reported recently in a murine obstructive airway disease model (28). The beneficial effects of CD4 activation may relate to the activation of regulatory CD4+ T cells, critical for long-term graft survival and tolerance, particularly after transient immunosuppressive protocols. In the absence of immunosuppression, alloreactive lymphocytes do recover or generate de novo. Regulatory T cells are the main force at this stage to control these aggressive cells.

In conclusion, by demonstrating selective inactivation of alloreactive CD8+ T cells as the consequence of disrupting the CD154-CD40 costimulation pathway that prevents accelerated rejection, this study provides new insights into divergent costimulatory requirements for T cell subsets. Our data are consistent with the key role of CD154 signaling in the mechanism of host sensitization to alloantigen.

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References