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Allograft Rejection by Primed/Memory CD8⁺ T Cells Is CD154 Blockade Resistant: Therapeutic Implications for Sensitized Transplant Recipients

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We have shown that CD8⁺ CTLs are the key mediators of accelerated rejection, and that CD8⁺ T cells represent the prime targets of CD154 blockade in sensitized mouse recipients of cardiac allografts. However, the current protocols require CD154 blockade at the time of sensitization, whereas delayed treatment fails to affect graft rejection in sensitized recipients. To elucidate the mechanisms of costimulation blockade-resistant rejection and to improve the efficacy of CD154-targeted therapy, we found that alloreactive CD8⁺ T cells were activated despite the CD154 blockade in sensitized hosts. Comparative CD8 T cell activation study in naive vs primed hosts has shown that although both naive and primed/memory CD8⁺ T cells relied on the CD28 costimulation for their activation, only naive, not primed/memory, CD8⁺ T cells depend on CD154 signaling to differentiate into CTL effector cells. Adjunctive therapy was designed accordingly to deplete primed/memory CD8⁺ T cells before the CD154 blockade. Indeed, unlike anti-CD154 monotherapy, transient depletion of CD8⁺ T cells around the time of cardiac engraftment significantly improved the efficacy of delayed CD154 blockade in sensitized hosts. Hence, this report provides evidence for 1) differential requirement of CD154 costimulation signals for naive vs primed/memory CD8⁺ T cells, and 2) successful treatment of clinically relevant sensitized recipients to achieve stable long term graft acceptance. The Journal of Immunology, 2002, 169: 4667–4673.

Host sensitization to a broad range of donor MHC Ags from multiple blood transfusions, prior failed grafts, or pregnancies remains one of the most critical problems in clinical organ transplantation (1, 2). Indeed, many sensitized patients are either precluded from receiving a transplant or may experience an increased rate of early rejection episodes that are irreversible or difficult to treat with current immunosuppressive agents. More basic studies to better appreciate the complex immune mechanisms in host sensitization to alloantigens are warranted. These, in turn, should lead to the development of much needed novel therapeutic approaches for the management of sensitized transplant patients.

We have long been interested in developing new strategies to manage sensitized transplant recipients, and over a decade ago established a rat model in which skin graft-induced sensitization triggers accelerated (24–36 h) rejection of vascularized cardiac allografts (3). As distinct from hyperacute rejection, which develops within minutes or hours and associates with high levels of pre-formed Abs to the donor, resulting in a classical picture of interstitial hemorrhages/vascular thrombosis, studies of accelerated rejection have suggested that both T and B cell responses may be important (1, 3, 4). We have recently developed a model of accelerated rejection of cardiac allografts in sensitized mice to further dissect the cascade of host cellular and humoral immune responses and to exploit a broader range of mAbs and the multitude of genetically deficient strains available (5). Although multiple effector pathways are involved, our studies have clearly shown that T cell-dependent mechanisms alone can mediate accelerated (<48 h) rejection of cardiac allografts.

T cell activation is dependent on interlocked signaling events that occur through engagement of cell surface receptors. In addition to the recognition of the MHC peptide complex by the TCR, efficient T cell activation and subsequent induction of effector functions require a second costimulation signal (6, 7). It is now generally accepted that CD40-CD154 interactions may provide such a signal during T cell activation, T cell-B cell interactions, and endothelial activation (8). CD154 (CD40 ligand), a member of the TNF family, is expressed predominantly on mature activated CD4⁺ and some CD8⁺ T cells, and interacts with CD40 expressed by APCs and B cells (9–11). Although the importance of CD154-mediated CD4⁺ T cell interactions with B cells is well recognized, it has been less clear what role CD154 may play in CD8⁺ T cell activation (12). The accruing data suggest that CD154-CD40-mediated activation of dendritic cells (DCs) by Ag-specific CD4⁺ T cells is essential for subsequent priming of CD8⁺ T cells (13–15). Thus, with T cell help needed to generate a productive CD8 T cell response, triggering of DCs by CD4 T cells was required before DC encounter with Ag-specific CD8⁺ T cells. Although activated CD8⁺ T cells can express CD154 (16, 17), little is known of putative functional roles and feedback interactions between CD8⁺ T cell CD154 and CD40 expressed by APCs. Recent evidence points toward a critical role of CD154 in the optimal generation of the mucosal CD8⁺ T cell response (18).

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 (19–21). This highlights the role of the CD40-CD154 costimulation pathway in the

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2 Abbreviations used in this paper: DC, dendritic cell; CTL, CTL effector; KO, knockout; MST, mean survival time; WT, wild type.
immune cascade leading to acute allograft rejection. However, relatively little is known of the role of CD154 costimulatory signals in the mechanism of host sensitization and whether their blockade may affect graft rejection in sensitized hosts. By employing CD154−/− mice as recipients of sequential skin and cardiac allografts and by targeting CD154−/− interactions in wild-type (WT) skin-sensitized hosts, we have recently documented an essential role of the CD154 pathway in host sensitization to alloantigen and identified CD8+ T cells as principal targets of the CD154 blockade in that model (22). Unlike preventive anti-CD154 mAb treatment in the sensitization phase (i.e., between skin and cardiac engraftment), peri-transplant CD154 blockade during the effector phase (i.e., at the time of cardiac engraftment) failed to protect heart transplants from accelerated rejection. The present study was aimed at dissecting putative mechanisms of CD154 costimulation blockade-resistant allograft rejection and improving the efficacy of this novel therapeutic strategy in sensitized recipients. We analyzed CD8+ T cells, principal targets of the CD154 blockade, in their costimulation requirements for activation in either naive or Ag-primed states. To the best of our knowledge, this study is the first to provide evidence for 1) differential requirement of CD154 costimulation signals for naive vs primed memory CD8+ T cells, and 2) successful treatment of sensitized recipients by adjunctive CD154 costimulation blockade protocols to achieve stable and long term graft acceptance in a clinically relevant transplantation model.

Materials and Methods

Animals

WT BALB/c (B/c; H-2d), B6/129 (B6; H-2d), CBA/Ca (CBA; H-2k) male mice and CD154-deficient (CD154−/−; B6) male mice (intercrossed at least 10 generations), aged 8–12 wk (20–25 g), were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the University of California, Los Angeles animal facilities under specific pathogen-free conditions.

Transplantation

Orthotopic full-thickness skin grafts (~0.5 cm in diameter) from B/c donors were sutured bilaterally onto the flanks of prospective WT or CD154−/− B6 recipients. These recipients were then challenged 10 or 40 days later with intraabdominal heterotopic B/c heart transplants. 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 selective pathological examination.

Treatment

Anti-mouse CD154 mAb (MR1; Bioexpress, West Lebanon, ME) was administered at cardiac engraftment (0.5 mg/mouse i.v.). Control recipients were treated with relevant doses of hamster Ig. Rat anti-mouse depleting CD8 (2.43; courtesy of Dr. H. Auchincloss, Massachusetts General Hospital, Harvard Medical School, Boston, MA) or CD4 (GK1.5, BD Pharmingen, San Diego, CA) was administered at either 0.25 (CD8) or 0.5 (CD4) mg/mouse/day, respectively, i.e. for 3 consecutive days (days −2, −1, and 0 of cardiac engraftment). Control animals were given relevant doses of rat Ig. In CTL induction experiments in vivo, MR1 (0.5 mg/mouse) or murine CTLA4Ig (0.25 mg/mouse) was used i.v. at the time of skin grafting.

In vitro MLR

Spleens were removed and passed through a cytospin screen 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% NH₄Cl and 5 mM Tris buffer, pH 7.2) at 37°C to lyse RBC. After washing with RPMI 1640/1% FBS, cells were suspended in culture medium (RPMI 1640 supplemented with 20 mM HEPES, 10 mM sodium pyruvate, 2 mM l-glutamine, 50 mM β-mercaptoethanol, 1× MEM-essential amino acid solution, 1× MEM-vitamin solution, 1× antibiotic/antimycotic solution, and 10% FBS) at a concentration of 5 × 10⁶/ml. One hundred microliters of responder B6 cells were added to a U-bottom 96-well plate (Corning, Corning, NY), mixed with the same number of gamma-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 μg/ml) was used as a positive control. One microcurie of [3H]Thymidine was added to each well in the last 16–18 h of a 3-day culture. Labeled cells were harvested onto filter membranes (Skatron Instruments, Sterling, VA) with a Skatron 12-well cell harvester. The counts per minute of the filter membrane were measured in scintillation liquid (Cytosport) on Beckman LS 6000IC.

For the proliferation analysis of T cell subsets, responder (B6) cells were labeled with CFSE (Molecular Probes, Eugene, OR) at 4 mM in PBS for 15 min at 37°C. The unconjugated CFSE was eliminated by washing the cells with FBS (20%)-supplemented RPMI 1640. The labeled cells were resuspended in culture medium and incubated with irradiated B6 (syngeneic), B/c (donor-type), or CBA (third-party) stimulator cells (2 × 10⁶/ml). On day 4 cells were harvested and stained with anti-mouse CD3e-R-PE (clone 145-2C11), CD4-biotin (clone H129.19), and streptavidin-Cy-Chrome (BD Pharmingen). 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). Cells in lymphocyte gate, Topro 3 negative (viable cells), stained positively for CD3 were analyzed for CFSE intensities (CD3+CD4− as CD4+; CD3+ as CD8+).

CTL effector differentiation in vivo

RBC-free splenocytes were prepared as described above. One million cells were used for Ab staining in ice-cold PBSA (PBS with 1% BSA). Cells were incubated with 10 μg normal rat IgG to block Fc binding sites. After washing, the cells were stained with 0.5–1 μg anti-mouse CD8α-FITC (clone 53-6.7), CD62L-R-PE (clone MEL-14), and CD44-Cy-Chrome (clone IM7, BD Pharmingen). The stained cells were washed, and three-color flow cytometry was performed on a FACScan cytometer. Cells in lymphocyte gate positive for CD8α were analyzed for CD26L and CD44 expression. CTL effectors (CTLe) were identified as the CD8+CD26−L−CD44− population (23).

In vitro CTL assay

B6 mouse splenocytes were obtained and cultured in bulk against gamma-irradiated donor B/c splenocytes for 6 days. Viable lymphocytes were counted and set up against 3H-Iradiated B/c target cells (Con A blast from B/c) at the ratio of 25 to 1. After 6-h incubation, supernatants were harvested and measured for gamma activity. Supernatants from wells of the target cells alone were counted as spontaneous release, and those from wells of target cell with 25% Triton 100 as maximal release. The specific cytotoxicity was calculated as % lysis = (cpm samples − cpm spontaneous)/cpm max − cpm spontaneous).

Statistical analysis

Comparisons were made using unpaired two-tailed Student’s t test. Differences in values at p < 0.05 were considered significant.

Results

CD154 blockade fails to affect allograft rejection in sensitized recipients

We have shown that although B/c hearts are rejected in 7 ± 2 days (mean survival time (MST) ± SD) in naive WT B6 mice, they are lost in an accelerated manner within 1.4 ± 0.3 days in B6 mice sensitized at day −10 with donor-type (B/c) skin grafts (5). Although the humoral immune response is involved, cell-mediated mechanisms play a dominant role in the accelerated rejection cascade in this model (5). Indeed, neutrophils contribute 50–60% of cells, with the remainder comprised of macrophages (~23%) and CD4 (~14%) and CD8 (~8%) T cells; NK and B cells contribute <1% of the graft infiltrate, whereas IL-2R expression is noted on some 15% of leukocytes. Moreover, RNase protection assay analysis showed that accelerated rejection is associated with intragraft mRNA expression of a number of chemokines (and their receptors), including macrophage inflammatory protein-1α (CCL1, CCR5); monocyte chemoattractant protein-1 (CCL2); RANTES and macrophage inflammatory protein-1β (CCL5); inducing protein-10 (CXCR3); plus the neutrophil chemokine receptor,
CXCR2; and the pan-leukocyte chemokine receptor, CXCR4. Rejection in this model is also accompanied by intragraft expression of the key T cell-associated cytokines, IFN-γ, plus IL-6 and IL-10.

Treatment of WT hosts with anti-CD154 (MR1) mAb every second day between the day of skin (day −10) and the day of cardiac (day 0) engraftment prevents host sensitization and results in long term (>100 days) cardiac graft acceptance with features of transplantation tolerance (22). To analyze whether CD154 blockade can successfully prevent graft rejection in sensitized hosts, we administered MR1 mAb on the day of cardiac engraftment (day 0). Unlike in naive mice (Fig. 1A), a single dose of MR1 mAb was only marginally effective (MST ± SD, 4 ± 1 days) in 10-day sensitized mice (Fig. 1B), suggesting that CD154 blockade is ineffective in preventing rejection in primed recipients.

Activated T cells triggered during a 10-day sensitization protocol might not be fully differentiated into memory-type cells at the time of cardiac engraftment. Thus, to distinguish between effector and memory lymphocytes, which may potentially be involved in the costimulation blockade-resistant rejection, we then refined our sensitized model in such a way that B6 mice were challenged with B/c hearts 40 day after donor-type skin engraftment. As shown in Fig. 1C, these long term sensitized recipients rejected B/c hearts in an accelerated, rather than acute, manner (MDT, 4 ± 1 days), and MR1 mAb treatment failed to affect graft survival (MST, 5 ± 1 days). Similar results were recorded with hearts transplanted up to 60 days after skin-induced sensitization. Thus, in marked contrast to the efficacy of CD154 blockade to prevent acute rejection in naive recipients, peri-transplant administration of anti-CD154 mAb failed to ameliorate accelerated rejection in sensitized mice.

CD154 blockade fails to affect activation of alloreactive CD8+ T cells in sensitized recipients

We have shown that CD8+ T cells are principal effectors in the accelerated rejection response, and the main targets of the CD154 blockade in presensitized recipients (22). The failure to inhibit alloreactive CD8+ T cells may represent a possible reason why rejection still occurs despite CD154 blockade in sensitized hosts. Thus, we contrasted the activation status of CD8+ T cells in sensitized mice with that in naive recipients. Spleen cells harvested on day 10 post-transplant from skin-sensitized or naive recipients of cardiac allografts treated with MR1 mAb on day 0 were analyzed for their alloreactivity in vitro MLR and cytotoxicity assays. By that time, presensitized recipients had already rejected their grafts, whereas naive recipients maintained well-functioning transplants.

As shown in Fig. 2A, splenocytes from sensitized hosts proliferated more vigorously against donor-type alloantigen compared with cells from naive hosts (p < 0.0005), but comparably against third-party Ags. FACS staining of CFSE-labeled splenocytes in bulk cultures against alloantigens has revealed vigorous proliferation of both CD4+ and CD8+ T cells in sensitized hosts, but selectively diminished CD8+ T cell proliferation in naive recipients (Fig. 2B). However, normal CD8+ T cells were able to proliferate if stimulated with Con A (comparable to rejecting CD8+ T cells; data not shown), indicating no overall CD8+ T cell defects in naive recipients. Thus, increased MLR counts in sensitized recipients resulted from the augmented proliferation of alloreactive CD8+ T cells. To confirm the activation of alloreactive CD8+ T cells, we also measured CD8-mediated cytotoxicity after 5–6 days of in vitro culture. Indeed, as shown in Fig. 3, stimulated cells from sensitized, but not naive, hosts effectively lysed donor-type target cells (75 vs 31% cytolysis, respectively, at E:T cell ratio of 50:1). Thus, CD154 blockade fails to prevent activation of alloreactive cytotoxic CD8+ T cells in sensitized mice.

Naive alloreactive CD8+ T cells are sensitive to CD154 blockade in vivo

Since CD154 blockade prevents allograft rejection in naive recipients, its failure in sensitized recipients raised the question of whether only naive, not primed, CD8+ T cells may require CD154 costimulation for their activation. Because of limited proliferation

FIGURE 1. Cardiac allograft survival. B/c hearts were transplanted heterotopically into either naive or skin-sensitized (as described in Materials and Methods) B6 mice. Anti-CD154 mAb (MR1) was administered on day 0 (i.e., at the time of cardiac engraftment). Graft survival was assessed daily by palpation of ventricular activity. There were four to six mice in each group.

FIGURE 2. A. MLR. Splenocytes were harvested on day 10 from either naive or skin-sensitized B6 mice that were treated with MR1 mAb on day 0. These cells (5 × 10^6/well) were cultured in vitro with the same number of irradiated stimulator splenocytes of B6 (syngeneic), B/c (donor-type), or CBA (third-party) strains in 96-well plates for 4 days. Cell proliferation was measured after pulsing the cells with [3H]Tdr for the last 16–18 h. The mean and SD are shown (representative of three independent experiments). B, Proliferation of T cell subsets. Splenocytes were labeled with CFSE as described. After culturing in vitro (2 × 10^6/ml) with the same number of irradiated stimulator splenocytes from B6 (syngeneic) or B/c (donor-type) strains for 4 days, cells were stained with anti-CD8-PE, anti-CD4-CyChrome, and Topo-3. Viable CD3+ lymphocytes (Topo-3 negative) were gated to analyze CFSE intensity profiles. The results of a representative experiment of a total of three performed are shown.
and differentiation of naive CD8$^+$ T cells in unseparated splenocytes in vitro against alloantigens, we monitored the activation of alloreactive CD8$^+$ T cells using an in vivo model system developed by Moby and Dailey (23, 24). In this model, B6 recipient mice were stimulated with B/c skin grafts. Indeed, as shown in Fig. 4, allogeneic, but not syngeneic, skin grafting triggered increased expression of splenic CD8$^+$ T cells with CD44$^{high}$CD62L$^{low}$ phenotype on day 10 (33 vs 3.7% in naive mice). These cells were functionally identified as activated alloreactive CD8$^+$ cytotoxic T cells. As shown in Fig. 4, CD154 blockade in naive B6 recipients at the time of skin grafting prevented the induction of CD8$^+$ cytotoxic T cell subset (2.8%). The CD154 requirement for naive CD8$^+$ CTL activation was also confirmed in the CD154 knockout (KO) system, in which allogeneic skin grafts failed to activate CD8$^+$ T cells (5.4%). Thus, CD154 costimulation signals are required for activation of naive alloreactive CD8$^+$ T cells.

**Activation of primed memory CD8$^+$ T cells was CD154 blockade resistant**

Primed alloreactive T cells consisted of either effector (day 10 after skin grafting) or memory (day 40 after skin grafting) cells in sensitized recipients. To address the question of whether these primed T cells may still require CD154 signaling when reactivated by allostimulation, we used in vitro and in vivo systems in parallel to monitor the activation of CD8$^+$ T cells from primed recipients in the absence or the presence of CD154 blockade. Splenocytes from skin-grafted recipients (10- or 40-day sensitization model) were first stimulated in vitro with alloantigen plus MR1 mAb for 5 days, followed by CD8$^+$ T cell differentiation/cytotoxicity assessment. As shown in Fig. 5A, CD154 blockade failed to prevent activation of primed effector/memory cytotoxic T cells, as evidenced by both differentiation of CTLs (31% of CD62L$^{low}$CD44$^{high}$ cells in total CD8$^+$ T cells in untreated or MR1-treated recipients) and effective lysis of target cells comparable with that of untreated T cells (Fig. 5B; 47 and 53% cytolyis at E:T cell ratio of 50:1 in MR1 mAb-treated and untreated recipients, respectively). However, unlike with CD154 blockade, CTLA4-Ig-mediated blockade of the CD28 costimulation pathway effectively inhibited activation of primed CD8$^+$ T cells. The percentage of CTLs decreased from 31% in untreated controls to 6.4% after CTLA4-Ig treatment (Fig. 5A), whereas cytolyis diminished from 53% in untreated controls to 36% in CTLA4-Ig-treated in vitro culture (Fig. 5B).

Since the requirement for CD154 signaling for naive CD8$^+$ T cell activation could only be addressed in vivo, we have modified our in vivo model system to measure the reactivation of primed CD8$^+$ T cells to directly compare the costimulation requirement of naive vs primed memory CD8$^+$ T cells. After the first (B/c) skin graft, recipient mice (B6) were rested for 40 days until the majority of
CD44\textsuperscript{high}CD62L\textsuperscript{low}CD8 cells returned to the naive CD62L\textsuperscript{high} phenotype state. As shown by Mobley et al. (24), CD44\textsuperscript{high}CD62L\textsuperscript{low}CD8\textsuperscript{+} T cells as well as CD44\textsuperscript{high}CD62L\textsuperscript{high} CD8\textsuperscript{+} T cells represent the population of memory alloreactive CD8\textsuperscript{+} T cells. With the second allogeneic skin challenge 40 days after the prime allograft, increased numbers of CD44\textsuperscript{high}CD62L\textsuperscript{low} CD8 cells reflect primarily activation of memory CD8\textsuperscript{+} T cells. Since CD154 blockade inhibits activation of naive CD8\textsuperscript{+} T cells, the frequency of the CD44\textsuperscript{high}CD62L\textsuperscript{low}CD8 subset should reflect only the memory CD8\textsuperscript{+} T cells. As shown in Fig. 6, there was a marked increase in the number of CD44\textsuperscript{high}CD62L\textsuperscript{low}CD8 cells triggered by the second skin graft (from 6.5 to 15.5%). CD154 blockade at the time of the second skin graft failed to inhibit the CD44\textsuperscript{high}CD62L\textsuperscript{low}CD8 increase (15.7% in MR1 mAb treated vs 15.5% in untreated controls). In contrast, and consistent with our in vitro data, CTLA4Ig-mediated blockade of CD28 effectively inhibited the induction of the CD44\textsuperscript{high}CD62L\textsuperscript{low} CD8 subset (6.4% in CTLA4-Ig treated vs 15.5% in untreated controls). Thus, activation of primed memory CD8\textsuperscript{+} T cells was CD154 blockade resistant.

**Adjunctive CD8 T depletion and CD154 blockade prolong allograft survival in sensitized hosts**

The presence of CD154 blockade-resistant CD8\textsuperscript{+} T cells prompted us to design adjunctive strategy to improve the efficacy of CD154 therapy in sensitized recipients. As there are no definitive markers suitable for selective depletion of memory/effector CD8\textsuperscript{+} T cells, we transiently depleted the whole CD8\textsuperscript{+} T cell population. Thus, B6 mice sensitized with B/c skin grafts (day −10 or −40) were treated from day −2 to 3 consecutive days with a depleting CD8 mAb, and then challenged with cardiac allografts (day 0), immediately followed by a single dose of MR1 mAb. Indeed, unlike control groups with intact CD8 subset plus MR1 mAb treatment (MST, 3.5 days) or CD8 depletion without MR1 mAb treatment (MST, 5.5 days), only adjunctive CD8 mAb plus CD154 mAb therapy resulted in long term graft acceptance (>50 days; n = 6) in the 10-day sensitization protocol (Fig. 7) and the 40-day sensitization protocol (>20 days; n = 4; not shown). Hence, CD8 T cell depletion represents a key prerequisite for therapeutically effective CD154 costimulation blockade in sensitized transplant recipients. As a control, adjunctive anti-CD4 mAb (days −2 to 0) plus anti-CD154 mAb therapy was also tested in sensitized mice. Unlike in the anti-CD8 mAb treatment group, transient depletion of CD4\textsuperscript{+} T cells had a marginal effect on the efficacy of subsequent CD154 blockade in sensitized recipients, with all cardiac allografts rejecting by 18 days (Fig. 7).

**FIGURE 6.** CTLA4 differentiation in primed B6 mice. Separate groups of B6 mice were challenged with B/c skin grafts (first skin graft) or with a subsequent set of skin grafts 40 days later (second skin graft). After receiving the second skin graft, recipients were treated with three doses of MR1, CTLA4-Ig, or control IgG (days 0, 2, and 4). Splenocytes were harvested 10 days following the second skin graft and stained with anti-CD8a-FITC, anti-CD62L-R-PE, and anti-CD44-CyChrome. CD8\textsuperscript{+} T cells were analyzed for CD62L and CD44 expression. CTLA4 were identified as CD8\textsuperscript{+} CD44\textsuperscript{high}CD62L\textsuperscript{low} (percentage in the CD8\textsuperscript{+} population). Representative data of three independent experiments are shown.

**FIGURE 7.** Cardiac allograft survival. B/c hearts were transplanted into donor-type skin-sensitized (day −10) B6 mice. The recipients were treated with MR1 alone (day 0; n = 4), depleting anti-CD8 mAb alone (days −2 to 0; n = 3), MR1 plus anti-CD8 mAb (n = 6), or MR1 plus anti-CD4 mAb (n = 4), as described in Materials and Methods. Graft survival was assessed daily by palpation of ventricular activity.

To analyze whether activation of alloreactive CD8\textsuperscript{+} T cells was indeed controlled long term in MR1 mAb-treated hosts after adjunctive CD8-targeted therapy, we examined the generation of CD8\textsuperscript{+} CTLe 40 days after heart transplantation. By that time, peripheral CD8\textsuperscript{+} T cells were recovered to ~50% from the transient depletion (Fig. 8A). Controls consisted of CD4 mAb- and MR1 mAb-treated mice examined 40 days after heart transplant, when CD4\textsuperscript{+} T cell recovery was close to 80%. Interestingly, as shown in Fig. 8B, newly generated CD8\textsuperscript{+} T cells in CD8/CD154 mAb-treated recipients bearing well-functioning grafts were well below the control level (<3% CD8\textsuperscript{+} CD44\textsuperscript{high}CD62L\textsuperscript{low} peripheral CD8\textsuperscript{+} T cells). In contrast, activated alloreactive CD8\textsuperscript{+} T cells were readily detectable in CD4/CD154 mAb-treated recipients.

**FIGURE 8.** T cell recovery and CD8 activation in long term surviving cardiac allograft recipients. Peripheral blood samples were obtained from B6 recipients 40 days after heart transplants. These mice were treated on day 0 with MR1 mAb plus a depleting anti-CD8 or anti-CD4 mAb, as described in Materials and Methods. Cells were stained with anti-CD4-CyChrome or anti-CD8-PE to measure T cell recovery or with anti-CD8a-FITC, anti-CD62L-R-PE, and anti-CD44-CyChrome to measure CD8 activation. Results are shown as FACS dot plots. One experiment, representative of three performed, is shown.
which did reject their grafts ~20 days earlier (~24% CD8\(^+\) CD44\(^++\)/CD62L\(^{low}\) in peripheral CD8\(^+\) T cells). As a single dose of MR1 mAb was given 40 days before that analysis, our findings indicate that initial CD154 blockade had a long-lasting suppressive effect on naive alloreactive CD8\(^+\) T cell development. In addition, CD154 blockade-resistant activation of primed CD8\(^+\) T cells may be CD4 help independent, as suggested by CD4 depletion controls.

**Discussion**

To establish a clinically relevant sensitized murine transplant model, we have modified the scheme of skin sensitization, cardiac transplantation, and therapeutic intervention based on our previous experience (5). Instead of preventive regimen starting on day −10, i.e., the day of a sensitizing skin graft challenge, we adapted peri-transplant treatment on day 0, i.e., the day of cardiac transplantation, by which time recipients are fully sensitized to alloantigen. To differentiate between effector and memory primed T cells, we further refined our model by sensitizing mice 40 days before cardiac engraftment. These short- and long-term sensitization schedules enable us to study effector/memory T cell responses against allografts and to elucidate how costimulation blockade may affect primed vs naive T cells.

Unlike preventive regimen, peri-transplant administration of MR1 mAb alone failed to affect accelerated rejection of cardiac allografts in both skin-sensitized (days −10 and −40) models. In addition, we detected alloreactive activated CD8\(^+\) CTLs in both types of the primed mice despite CD154 blockade at the time of cardiac engraftment. To explore putative mechanisms of CD154 blockade-resistant rejection and CD8\(^+\) T cell activation, we asked whether activation of alloreactive CD8\(^+\) T cells may have different costimulation requirements in naive vs primed environments. To address that issue, we first examined in vitro CD8\(^+\) T cell responses using splenocytes from naive or skin-sensitized B6 mice in the absence or the presence of MR1 mAb. Increased CD154 mAb-independent CD8\(^+\) T cell proliferation and differentiation against alloantigen were readily observed with primed T cells. Since naive CD8\(^+\) T cells responded less vigorously in vitro, and we were not sure whether ex vivo manipulation affects the functional state of splenocytes, we adapted (23, 24) and modified an in vivo model system to study alloreactive CD8\(^+\) T cell activation. Direct measurement of recently activated CD8\(^+\) T cells in the periphery from skin-grafted WT mice treated with anti-CD154 mAb or from untreated CD154 KO mice revealed that CD154 signals are indeed required for activation of naive alloreactive CD8\(^+\) T cells. However, restimulation with the same donor-type skin graft in primed mice augmented CTL differentiation/cytolysis despite concomitant anti-CD154 mAb therapy. This indicates the emergence of CD154 costimulation-independent activation of CD8\(^+\) T cells in primed mice, representing most likely either effector or memory CD8\(^+\) T cell generation. Spleen T cells from these mice triggered accelerated rejection of cardiac allografts after infusion into otherwise normal recipients despite concomitant MR1 mAb treatment, documenting that CD154 blockade-resistant CD8\(^+\) primed/memory T cells were transferable (Y. Zhai, unpublished observations). Interestingly, both naive and memory/effector CD8\(^+\) T cells require CD28 costimulation signaling for their activation, which indicates the functional hierarchy of costimulation molecules in T cell activation. Indeed, we have previously shown the efficacy of CTLA4-Ig therapy to prevent allograft rejection in sensitized rats (25). Moreover, consistent with our current CD8 activation data, the effect of CD28 blockade on cardiac allograft survival in skin-sensitized mice (day −10 model) in our ongoing studies was superior to that of CD154 blockade (MST, ~12 and 2 days, respectively).

We then attempted to address the functional importance of these costimulation blockade-resistant primed memory CD8\(^+\) T cells in the allograft rejection cascade. Although adjunctive infusion of a depleting anti-CD8 depleting mAb into already sensitized recipients eliminates all CD8\(^+\) T cells, its additional therapeutic effect relative to CD154 monotherapy was targeted mainly at the primed CD8\(^+\) T cell, because the CD154 blockade alone was sufficient to control the naive CD8\(^+\) T cell population. The prolongation of graft survival after combined peri-transplant CD8 depletion and CD154 blockade stresses the importance of primed CD8\(^+\) T cells in costimulation blockade-resistant rejection. To the best of our knowledge, this study is the first to document the differential requirement of CD154 costimulation signals for activation of naive vs primed (effector/memory) CD8\(^+\) T cells.

This study complements our recent findings on the key role of alloreactive CD8\(^+\) T cells as the prime targets of CD154-targeted therapy and effectors in the immune rejection cascade in sensitized transplant recipients (22). The issue of whether alloreactive CD8\(^+\) T cells represent the targets for CD154 blockade in transplant recipients has been controversial. It was hypothesized that CD4-dependent CD8\(^+\) T cell activation may rely on CD154 costimulation signals that activate DCs (13–15). In our case, however, alloreactive CD8\(^+\) T cell activation in vivo was both CD4 dependent and independent, as CD4\(^+\) T cell depletion or the CD4 KO environment decreased, but it did not completely abolish, CD8\(^+\) T cell activation (Y. Zhai, L. Meng, R. W. Busuttil, M. H. Sayegh, and J. W. Kupiec-Weglinski, manuscript in preparation). Thus, we favor the idea that there is a CD4-independent, CD154-dependent pathway for alloreactive CD8\(^+\) T cell activation. A similar conclusion has been drawn from allogeneic murine tumor models (26, 27). In addition, it has been shown that long term expansion of adoptively transferred 2C TCR Tg CD8\(^+\) T cells in Ag-bearing F1 hosts depends on CD154 costimulation signals via CD4\(^+\) T cells (28). It has also been documented, however, that intragraft proliferation, homing, and cytokine production by Tg-TCR\(^+\) (from BM3 or DES mice) CD8\(^+\) T cells was CD154 blockade independent (29). In a multiple minor histocompatibility Ag-mismatched skin allograft model, CD154 blockade was more effective in preventing CD4-mediated than CD8-mediated rejection in WT recipients (30). It delayed CD8\(^+\) T cell-mediated rejection, resulting in significant prolongation of graft survival rather than its permanent acceptance. In this case, activation of CD8\(^+\) T cells was CD4\(^+\) T cell dependent. In an intestinal transplant model, CD154 blockade exerted some therapeutic effect in CD8 KO, but not in CD4 KO, recipients (31). Perhaps, the efficacy of CD154 blockade to target CD4 or CD8 T cell subsets may be a quantitative issue, depending on the downstream events after CD4/CD8 T cell activations in the processes of graft rejection. More importantly, CD8\(^+\) T cells do not represent a homogenous population; susceptibility or resistance to the CD154 blockade may not necessarily apply to the entire population, but rather to a specific T cell subset. Indeed, the asialo-GM1 CD8\(^+\) T cell subset was shown to be responsible for the rejection of allogeneic skin grafts despite simultaneous CD28 and CD154 costimulation blockade (32). In addition, CD40-independent pathways of CD4\(^+\) T cell help for priming of CD8\(^+\) CTLs were demonstrated in a TCR Tg model (33).

Our results support the role of CD154 costimulation signals in activation of alloreactive CD8\(^+\) T cells, but limit its effect on the naive CD8\(^+\) T cell population. Importantly, we have determined that both effector as well as memory T cells may be potentially CD154 blockade resistant. This adds an additional level of complexity to distinct roles of T cell costimulation pathways in CD8\(^+\) T cells.
T cell activation. Indeed, costimulation requirements may vary under different physiological or experimental conditions with known variables, including Ag types or doses (34, 35), the precursor frequency of responding cells (36), other stimulating help (e.g., cytokines) (37–39), as well as functional T cell status (40).

Our finding that primed memory cells CD8+ T cells have differential requirements for CD154 costimulation compared with their naive counterparts is of clinical significance in improving the efficacy of current costimulation blockade-based therapeutic regimens. The priming of alloreactive CD8+ T cells occurs not only during allograft transplantation or following multiple blood transfusions, but may also result from viral or bacterial infections as well as other immune reactions due to the broad cross-reactivity of alloreactive T cells (41, 42). In the latter case, the frequency of primed T cells may not be as high as that triggered by allogeneic stimulation. However, these CD8+ T cells may well escape the CD154 blockade and then contribute to subacute or chronic-type rejection episodes. Therefore, as the CD154 blockade fails to completely control alloreactive CD8+ T cells, adjunctive targeting of CD8+ T cells to optimize therapeutic effects in transplant recipients may be warranted.

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References