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Activation of Alloreactive CD8⁺ T Cells Operates Via CD4-Dependent and CD4-Independent Mechanisms and Is CD154 Blockade Sensitive

Yuan Zhai,* Lingzhong Meng,* Ronald W. Busuttil,* Mohamed H. Sayegh,† and Jerzy W. Kupiec-Weglinski²*

CD154, one of the most extensively studied T cell costimulation molecules, represents a promising therapeutic target in organ transplantation. However, the immunological mechanisms of CD154 blockade that result in allograft protection, particularly in the context of alloreactive CD4/CD8 T cell activation, remain to be elucidated. We now report on the profound inhibition of alloreactive CD8⁺ T cells by CD154 blockade via both CD4-dependent and CD4-independent activation pathways. Using CD154 KO recipients that are defective in alloreactive CD8⁺ T cell activation and unable to reject cardiac allografts, we were able to restore CD8 activation and graft rejection by adoptively transferring CD4⁺ or CD8⁺ T cells from wild-type syngeneic donor mice. CD4-independent activation of alloreactive CD8⁺ T cells was confirmed following treatment of wild-type recipients with CD4-depleting mAb, and by using CD4 KO mice. Comparable levels of alloreactive CD8⁺ T cell activation was induced by allogenic skin engraftment in both animal groups. CD154 blockade inhibited CD4-independent alloreactive CD8⁺ T cell activation. Furthermore, we analyzed whether disruption of CD154 signaling affects cardiac allograft survival in skin-sensitized CD4 KO and CD8 KO recipients. A better survival rate was observed consistently in CD4 KO, as compared with CD8 KO recipients. Our results document CD4-dependent and CD4-independent activation pathways for alloreactive CD8⁺ T cells that are both sensitive to CD154 blockade. Indeed, CD154 blockade was effective in preventing CD8⁺ T cell-mediated cardiac allograft rejection. The Journal of Immunology, 2003, 170: 3024–3028.

To become fully activated, T cells require two distinct signals. The first, which is Ag specific, derives from TCR complex interacting with MHC-peptides on APCs. The second, provided by Ag-nonspecific costimulation, includes a large variety of cell surface molecules (1, 2). Costimulation has been demonstrated to be essential for the complete activation of T cells, in driving cell proliferation (clonal expansion) (3, 4), reducing apoptosis, and promoting effector cell differentiation (5), as well as in memory cell formation (6). CD154 (CD40 ligand), a type II membrane protein of the TNF superfamily, represents one of the best-characterized costimulation molecules (7). Its expression is induced upon partial activation of CD4⁺ T cells and possibly some CD8⁺ T cells. The interaction of CD154 with CD40 is pivotal for CD4⁺ T cell costimulation as well as CD4⁺ T cell help for B cells and CD8⁺ T cells. Ag-presenting dendritic cells (DC)³ may bridge the CD4 help to CD8⁺ CTLs via the CD154-CD40 costimulation pathway (8, 9).

In organ transplantation, T cell costimulation blockade is one of the most promising therapeutic strategies developed in the past two decades. The efficacy of CD154-targeted therapy to abrogate the rejection response and to markedly prolong allograft survival in rodents and subhuman primates has now been well established (10, 11). This highlights the role of CD40-CD154 costimulation pathway in the immune cascade leading to allograft rejection. However, the immunological mechanisms that mediate in vivo effects of CD154 costimulation blockade in allogeneic transplant models remain controversial, particularly in regard to alloreactive CD8⁺ T cells. Although CD154 blockade effectively inhibited primary alloreactive CD4⁺ T cell responses in a number of transplant models, it was relatively ineffectual in blocking alloreactive CD8⁺ T cell activation (12–15). In fact, alloreactive CD8⁺ T cells were ascribed as the major contributors of costimulation blockade-resistant rejection. However, CD8⁺ T cells did indeed require CD154 costimulation for their activation in allogeneic MHC class I tumor models (16). Additionally, by using CD4-deficient mice, it was shown that antitumor CD8⁺ T cell responses operate via a CD4-independent pathway, and are susceptible to CD154 blockade (17). As disparate immune mechanisms are involved in the activation of MHC class I-disparate allogeneic tumors vs MHC fully mismatched allogeneic organ transplants, the validity of this finding needs to be tested in well-defined transplantation models.

While studying antiviral CD8⁺ T cell responses, it became clear that the activation of Th-independent CD8⁺ T cells was CD154 independent, whereas activation of Th-dependent CD8⁺ T cells relied on CD154 costimulation to generate efficient CTL responses (18). Alloreactive T cells (both CD4 and CD8) may recognize alloantigen in a unique way, such that TCRs are able to interact
isolated CD4 (Figs. 1 and 2), we also used enriched T cells from CD8 KO mice as way in host sensitization to alloantigen, and identi hosts, we have recently documented the key role of CD154 path CD154-CD40 interactions in wild-type (WT) skin-sensitized be elucidated.

By employing CD154-deficient (knockout (KO)) mice as recipients of sequential skin and cardiac allografts, and by targeting CD154-CD40 interactions in wild-type (WT) skin-sensitized hosts, we have recently documented the key role of CD154 path CD154-CD40 interactions in wild-type (WT) skin-sensitized be elucidated.

Materials and Methods

Animals and grafting techniques

WT BALB/c (H-2b), C57BL/6 (H-2b), and CBA/Ca (H-2b) male mice were used. We also used mice deficient of CD154 (CD154 KO), CD40 (CD40 KO), CD4 (CD4 KO), and CD8 (CD8 KO) of B6 background (intercrossed at least 10 generations). All mice (age of 8–12 wk; 20–25 g) were obtained from The Jackson Laboratory (Bar Harbor, ME), and were housed in the University of California, Los Angeles, animal facilities under pathogen-free conditions. Orthotopic full-thickness skin grafts (~0.5 cm in diameter) from BALB/c donors were sutured bilaterally onto the flanks of prospectiv B6 recipients. Some of these were then challenged 10 days later in an intra-abdominal location with vascularly anastomosed BALB/c hearts. 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.

T cell subset isolation and adoptive cell transfers

T cells were enriched using nylon wool columns (10 ml; Polysciences, West Lebanon, ME), was administered at the time of skin transplan-

Ab therapy

Rat anti-mouse CD4 (GK1.5) and CD8 (2.43; courtesy of Dr. H. Auchin-}

CTL effector differentiation in vivo

RBC-free lymphocytes from PBL, splenocytes, or lymph node cells were prepared. 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 IgG3 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), CD62L-PE (clone MEL-14), and CD44-CyChrome (clone IM7) (BD Pharmingen, San Diego, CA). After washing, three-color flow cytometry was performed on a FACSScan cytometer (BD Biosciences, Mountain View, CA). Cells in lymphocyte gate and stained pos-

In vitro CTL assay

B6 mouse splenocytes were obtained and cultured in bulk against gamma-irradiated donor BALB/c splenocytes for 6 days. Viable lymphocytes were counted and set up against 51Cr-labeled BALB/c target cells (Con A blast from 3- to 4-day cultures) in a U-bottom 96-well plate at different ratios. After a 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 X-100 as maximal release. The specific cytosis was calculated as follows: % = (cpm samples - cpm spontaneous)/cpm max - cpm spontaneous).

FIGURE 1. Cardiac allograft survival in skin-sensitized CD154 KO recipients. Fifty to 100 million splenocytes from distinct B6 donors were adoptively transferred i.v. into CD154 KO mice. BALB/c skin grafts were placed on the day of cell transfer, followed 10 days later by BALB/c hearts. The cardiac allograft survival data was charted with the following recipient groups: WT, WT B6 recipient control (MST ± SD = 1.5 ± 1 days, n = 10); CD154 KO recipient control (MST > 50 days, n = 10); +WT Sp, CD154 KO recipient infused with 5 × 10^5 WT splenocytes (MST ± SD = 4 ± 1 days, n = 4); +CD4 KO Sp, CD154 KO recipient infused with 5 × 10^5 splenocytes from CD40 KO donors (MST ± SD = 4.5 ± 1 days, n = 4); and +nu/nu Sp, CD154 KO recipient infused with 10 × 10^7 splenocytes from nu/nu mice (MST > 50 days, n = 4).

FIGURE 2. Cardiac allograft survival in skin-sensitized CD154 KO recipients. Fifty million unseparated splenocytes or five million highly purified T cells from distinct B6 donors were adoptively transferred i.v. into CD154 KO recipients. BALB/c skin grafts were placed on the day of cell transfer, followed by a BALB/c heart 10 days later. The cardiac allograft survival data was charted with the following groups of recipients: WT, WT B6 recipient control (MST ± SD = 1.5 ± 1 days, n = 10); CD154 KO recipient control (MST > 50 days, n = 10); +CD4+, CD154 KO recipient infused with 5 × 10^7 splenocytes from CD8 KO mice or five million highly purified CD4^+ T cells (MST ± SD = 6.7 ± 3 days, n = 4); +CD8^+, CD154 KO recipients infused with 5 × 10^7 splenocytes from CD4 KO mice or five million highly purified CD8^+ T cells (MST ± SD = 7.5 ± 1 days, n = 4); and -T, CD154 KO recipients infused with 10 × 10^7 T cell-depleted splenocytes from WT B6 donors (MST > 50 days, n = 4).
FIGURE 3. Alloreactive CD8^+ T cell activation in skin-sensitized adoptively transferred CD154 KO recipients. Blood samples were collected from the tail vein of the groups of recipients, as in Fig. 2, and stained for CD8, CD44, and CD62L, as described in Materials and Methods. FACS density plots are shown based on CD8^+ gating with CD62L staining as x-axis, and CD44 staining as y-axis. The percentages represent the percentages of CD8^+ cells with the CD44^{hi}CD62L^{lo} phenotype. These data are representative of at least four individual recipients per group.

Results

CD154 costimulation pathway activates alloreactive CD8^+ T cells via both CD4-dependent and CD4-independent mechanisms

We have previously shown that alloreactive CD8^+ T cell activation is inhibited in CD154 KO mice (19). This finding provided us with an adoptive cell transfer model system to elucidate putative components in the CD154 costimulation pathway that may lead to activation of alloreactive CD8^+ T cells. First, we used splenocytes from groups of WT, nu/nu, and CD40 KO syngeneic B6 mice to determine whether the defect in alloreactive CD8^+ activation in CD154 KO mice was at the T cell or APC side. Fifty million cells were infused i.v. into CD154 KO mice, followed by a BALB/c skin graft on the same day. Ten days later, a BALB/c cardiac graft was transplanted into these skin-primed recipients. Alloreactive CD8^+ T cell activation was examined by blood sampling at day 10, and allograft rejection was monitored by daily palpation. As shown in Fig. 1, CD154 KO recipients reconstituted with splenocytes from WT or CD40 KO mice rejected their cardiac allografts promptly (mean survival time (MST) ± SD = 4 ± 1 and 4.5 ± 1 days, respectively), whereas those reconstituted with cells from nu/nu mice (up to 100 million) did not (MST ≥ 50 days). This indicates that the defect that prevented alloreactive CD8^+ T cell activation in CD154 KO mice was T cell- rather than APC-dependent, because APCs from nu/nu mice that were competent of delivering CD154 costimulation to T cells failed to restore CD8^+ T cell-mediated allograft rejection, while T cells from CD40 KO mice that were competent of receiving but not delivering CD154 signaling readily restored transplant rejection.

Next, we used highly purified T cell subsets from WT mice or unseparated splenocytes from CD4 KO or CD8 KO mice in our transplantation model. Because the non-T cell fraction of WT splenocytes or splenocytes from nu/nu mice all failed to affect rejection in CD154 KO recipients (Fig. 2), we reasoned that splenocytes from CD4 and CD8 KO mice were functionally similar to isolated CD8^+ and CD4^+ T cells, respectively. Thus, five million isolated CD4^+ or CD8^+ T cells or 50 million splenocytes from CD4 or CD8 KO mice were infused into CD154 KO recipients of cardiac allografts, as in previous experiments. CD154 KO recipients reconstituted with CD4^+ or CD8^+ T cells alone were able to reject their cardiac allografts (MST ± SD = 6.7 ± 3 and 7.5 ± 1, respectively; Fig. 2). Activation of alloreactive CD8^+ T cells was also detected in these reconstituted hosts, although not up to the levels seen in WT recipients (23 and 22% in CD4^+ and CD8^+ T cell-reconstituted CD154 mice, respectively; Fig. 3). Because no CD154^+CD8^+ T cells were present in CD154 KO mice reconstituted with CD4^+ T cells, the restoration of alloreactive CD8^+ T cell activation indicated that adoptively transferred CD154^+CD8^+ T cells provided help to CD154^+CD8^+ T cells. Additionally, because there were no CD154^+CD4^+ T cells to provide help to CD154^+CD8^+ T cells in CD154 KO mice reconstituted with CD8^+ T cells, CD8^+ T cells provided help for their own activation. These results clearly demonstrate the existence of two distinct CD154 costimulation pathways that operate via either CD4-dependent or CD4-independent mechanisms to activate alloreactive CD8^+ T cells.

CD4-independent CD8^+ T cell activation requires CD154 costimulation

CD4-dependent CD154 costimulation for CD8^+ T cell activation was initially demonstrated in antiviral model systems (8, 9). Alloreactive CD8^+ T cells are distinct from antiviral CD8^+ T cells in such a way that they can recognize intact alloantigen in their native forms. An extremely high precursor frequency is associated with those alloreactive T cells in the direct allore cognition pathway. Hence, in addition to the above in vivo cell reconstitution experiments, we performed in vitro CTL assays to confirm CD4-independent CD8^+ CTL activation. T cells from groups of WT and CD4 KO recipients of BALB/c skin grafts were stimulated in vitro in MLRs, and CD8-mediated cytotoxicity against BALB/c Con A blasts was then measured 5 days later. Indeed, as shown in Fig. 4, unlike cells from naive WT or CD4 KO mice, CD8^+ T cells from engrafted WT and CD4 KO recipients were activated and were able to lyse target cells at comparable levels.

Because little or no CD154 expression was detected on αβ CD8^+ T cells (20, 21), the question as to whether CD154 costimulation pathway operates in CD4-independent CD8^+ T cell activation needs to be addressed. To eliminate the possibility that CD8 responses are aberrant in CD4 KO mice, we studied WT mice that were treated with anti-CD4 depleting mAb for 2 consecutive days prior skin grafting. FACS analysis confirmed CD8^+ T cell depletion, and CD8^+ T cell activation was assessed by surface phenotyping. As in untreated WT recipients, significant levels of allo-

FIGURE 4. CTL assay of in vivo-primed splenocytes. Splenocytes were harvested from either naive or BALB/c skin-primed WT or CD4 KO B6 mice and set up in vitro in MLRs against irradiated BALB/c splenocytes. After 5-day culture, CTL assays were performed against BALB/c Con A blasts, as described in Materials and Methods. Percentages of target cell cytolyis were charted against different effector/target ratios. These data are representative of three assays performed at different occasions.
CD154 blockade prevents CD8⁺ T cell-mediated allograft rejection

In this study, we focused on mechanisms of alloreactive CD8⁺ T cell activation, and our results show that both CD4 help-dependent and CD4 help-independent pathways operate and are sensitive to CD154 costimulation blockade. Our recent finding that alloreactive CD8⁺ T cell activation is defective in CD154 KO mice (19) prompted us to investigate the actual defect that prevents activation of CD8⁺ T cells in our transplantation model. Our present cell reconstitution experiments document the key defect at the T cell rather than the APC side. This result is consistent with the observation from allogenic tumor model in which anti-CD40 Ab, which activates APCs, was unable to substitute for CD154 in activating alloreactive CD8⁺ T cells (16). A similar phenomenon was observed in our model with IgM-type anti-CD40 Ab (data not shown). The fact that both T cell subsets from WT mice were able to recreate not only the activation of alloreactive CD8⁺ T cells but also cardiac allograft rejection mediated by these activated T cells, clearly points toward the existence of dual pathways of alloreactive CD8⁺ T cell activation. The CD4-independent activation of alloreactive CD8⁺ T cells was confirmed both in vivo with phenotypic changes by allostimulation and in vitro by cytotoxicity assays. The latter is of importance, because others have shown that T cells are the key elements in initiating the rejection cascade in this sensitized transplant model (22). Because BALB/c hearts were not rejected acutely in naive CD4 KO B6 recipients (graft survival > 30 days; data not shown), we used BALB/c skin grafts to trigger accelerated cardiac allograft rejection. Indeed, as shown in Fig. 6, skin-sensitized CD4 KO mice rejected donor-type heart grafts within 7 days. A single dose of anti-CD154 mAb at the time of skin grafting not only prevented BALB/c hearts from rejection (Fig. 6), but also protected BALB/c skins in CD4 KO recipients (data not shown). In marked contrast, anti-CD154 mAb in skin-sensitized CD8 KO mice prolonged the survival of cardiac allografts to ~50 days (Fig. 6), whereas donor-type skin grafts were still lost within 10 days (data not shown).

Discussion

In this study, we focused on mechanisms of alloreactive CD8⁺ T responses, we have established a skin-primed cardiac allograft rejection model in CD4 KO and CD8 KO mice. We have previously shown that T cells are the key elements in initiating the rejection cascade in this sensitized transplant model (22). Because BALB/c hearts were not rejected acutely in naive CD4 KO B6 recipients (graft survival > 30 days; data not shown), we used BALB/c skin grafts to trigger accelerated cardiac allograft rejection. Indeed, as shown in Fig. 6, skin-sensitized CD4 KO mice rejected donor-type heart grafts within 7 days. A single dose of anti-CD154 mAb at the time of skin grafting not only prevented BALB/c hearts from rejection (Fig. 6), but also protected BALB/c skins in CD4 KO recipients (data not shown). In marked contrast, anti-CD154 mAb in skin-sensitized CD8 KO mice prolonged the survival of cardiac allografts to ~50 days (Fig. 6), whereas donor-type skin grafts were still lost within 10 days (data not shown).

CD154 blockade prevents CD8⁺ T cell-mediated allograft rejection

To document the efficacy of CD154 blockade to prevent alloreactive CD8⁺ T responses, we have established a skin-primed cardiac allograft rejection model in CD4 KO and CD8 KO recipients. BALB/c skin grafts were placed onto CD4 KO or CD8 KO B6 recipients 10 days prior to BALB/c hearts. MR1 mAb was administered at the time of skin grafting (0.5 mg/mouse i.p.). The cardiac allograft survival data was charted with the following groups of recipients: CD4 KO/skin (MST ± SD = 5 ± 2 days, n = 3); CD8 KO/skin (MST ± SD = 6.5 ± 1 days, n = 3); CD4 KO/skin/MR1 (MST ± SD > 50 days, n = 4); and CD8 KO/skin/MR1 (MST ± SD = 45 ± 5 days, n = 3).

FIGURE 6. Cardiac allograft survival in skin-sensitized CD4 or CD8 KO recipients. BALB/c skin grafts were placed onto CD4 KO or CD8 KO B6 recipients 10 days prior to BALB/c hearts. MR1 mAb was administered at the time of skin grafting (0.5 mg/mouse i.p.). The cardiac allograft survival data was charted with the following groups of recipients: CD4 KO/skin (MST ± SD = 5 ± 2 days, n = 3); CD8 KO/skin (MST ± SD = 6.5 ± 1 days, n = 3); CD4 KO/skin/MR1 (MST ± SD > 50 days, n = 4); and CD8 KO/skin/MR1 (MST ± SD = 45 ± 5 days, n = 3).
million purified T cells did not have any measurable impact on these two parameters (data not shown).

The role of CD154 in the mechanism of CD8-mediated transplant rejection remains controversial. In contrast to previous reports (12–15), we have found that the effects of CD154 blockade in preventing allogenic skin and cardiac graft rejection were more profound while targeting CD8+ rather than CD4+ T cells. Indeed, not just cardiac allografts, but also allogenic donor-type sensitizing skin grafts survived long-term in CD4 KO recipients given a single dose of MR1 mAb. In contrast, in skin-sensitized CD8 KO recipients, most cardiac and skin allografts rejected within 50 and 20 days, respectively, despite the CD154 blockade. These data are consistent with results from studies in class I-disparate allogenic tumor models in which CD4-independent CTL induction was shown to be CD154 dependent. Originally, the efficacy of CD154 blockade to effectively suppress alloreactive CD4+ rather than CD8+ T cells was demonstrated in a murine graft vs host disease model (12). By adoptively transferring CD4+ or CD8+ T cells into irradiated allogenic test recipients, treatment with anti-CD154 mAb significantly prolonged the survival of bm12 recipients of B6 CD4+ T cells, but not of bm1 recipients of B6 CD8+ T cells. Similarly, alloreactive TCR-transgenic CD8+ T cells transferred into CBA hosts became activated, proliferated, and migrated to B10 cardiac allografts despite the CD154 blockade (14). Distinct recipient strains and transplant models might potentially contribute to the differential effects of CD154 blockade (24). We believe that the rejection mechanism involving different aspects of T cell function might be the key. In our model, we found that, although CD8+ T cells were unable to differentiate into effector cytotoxic T cells, they still produced cytokines (including IFN-γ, data not shown). Thus, if CD8 effectors were not solely responsible for rejection, other effector cells recruited by cytokines could eventually have joined the force to reject the transplant. In addition, adoptive transfer ex vivo manipulations might have also modulated host responsiveness to the CD154 blockade. As in our previous report (25), activated CD8+ T cells (effector or memory cells) but not naive T cells, did not require CD154 signaling for their activation.

The mechanism of CD4 help for CD8+ T cells via CD154 costimulation is well established. However, it is unclear how the activation of CD4-independent CD8+ T cells might operate via the CD154 pathway. As has been observed in at least two other cases, the CD154 molecule may be expressed transiently within a small subset of CD8+ T cells (21, 26, 27). Due to the extremely high precursor frequencies, some alloreactive CD8+ T cells that express CD154 molecules upon initial TCR-allo-MHC interaction may provide sufficient help to activate DCs. These DCs will further activate the majority of CD154-negative CD8+ T cells. Indeed, a notion of CD8-self help in the context of CD4 help, has been recently introduced in antiviral model systems (28, 29).

In summary, this study demonstrates the existence of dual pathways of alloreactive CD8+ T cell activation, and documents that both CD4-dependent and CD4-independent CD8+ T cell activation require CD154 costimulation. This complements our previous studies (19, 25) and further points toward the CD8+ T cell as the principle target of the CD154 costimulation blockade in organ transplantation.

References

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In summary, this study demonstrates the existence of dual pathways of alloreactive CD8+ T cell activation, and documents that both CD4-dependent and CD4-independent CD8+ T cell activation require CD154 costimulation. This complements our previous studies (19, 25) and further points toward the CD8+ T cell as the principle target of the CD154 costimulation blockade in organ transplantation.