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Different Costimulatory and Growth Factor Requirements for CD4\(^+\) and CD8\(^+\) T Cell-Mediated Rejection\(^1\)

Minh Diem Vu, \(^2\)* Farhana Amanullah, \(^2\)* Yongsheng Li, \(^*\) Gulcin Demirci, \(^*\) Mohamed H. Sayegh, \(^†\) and Xian Chang Li \(^3\)*

Costimulatory signals and growth factor signals play a key role in commanding T cell activation and T cell effector function. However, how costimulatory signals and growth factor signals interact and integrate into the activation program of CD4\(^+\) and CD8\(^+\) T cells during the allograft response remains poorly defined. In the present study we found that either CD4\(^+\)- or CD8\(^+\)-deficient mice can vigorously reject the skin allografts. Blocking rapamycin-sensitive growth factor signals produced long-term skin allograft survival in CD4\(^+\)-deficient mice (mean survival time, >120 days), but not in CD8\(^+\)-deficient mice (mean survival time, 20 days). Analysis of CFSE-labeled cells proliferating in the allogeneic hosts revealed that clonal expansion of CD4\(^+\) T cells in vivo was more resistant to growth factor blockade than that of CD8\(^+\) T cells. However, blockade or genetic absence of CD28/CD154 costimulatory molecules rendered CD4\(^+\) T cell-mediated rejection sensitive to rapamycin, and long-term skin allograft survival can be readily induced by rapamycin in the absence of CD28/CD154 signals (>100 days). Furthermore, blocking OX40 costimulation induced long-term skin allograft survival in CD4\(^+\)-deficient mice and CD8\(^-\)-deficient mice when both CD28 and CD154 were transiently blocked. We conclude that CD4\(^+\) and CD8\(^+\) T cells exhibit distinct sensitivity to growth factor blockade in transplant rejection, and CD28/CD154-independent rejection is sensitive to rapamycin and appears to be supported by OX40 costimulation. *The Journal of Immunology,* 2004, 173: 214–221.

Central to the events of allograft rejection are the activation and rapid clonal expansion of alloreactive T cells. It is well known that a productive T cell response is a highly coordinated interplay of multiple signals. TCR stimulation is required, but alone is not sufficient for robust T cell activation, and a second signal delivered by costimulatory molecules plays a critical role in this regard (1, 2). The fate of activated T cells to become armed effector T cells, to undergo apoptotic cell death, or to evolve as memory cells, however, is regulated primarily by T cell growth factors (TCGFs)\(^3\) (3), a family of cytokines with potent T cell mitotic activities (i.e., IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) (4, 5).

One of the key features in transplant rejection is the multiplicity of cell types involved and the complexity of their interactions and regulations. Indeed, activation of both CD4\(^+\) and CD8\(^+\) T cells is frequently observed in rejection of fully MHC-mismatched allografts (6). Adding to the complexity is the presence of multiple T cell costimulatory molecules (e.g., CD28, CD154, CD27, CD70, OX40 (CD134), 4-1BB (CD137), inducible costimulator, etc.) and diverse TCGFs (i.e., IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) involved in T cell activation (3, 7). It is often believed that activation of naive CD4\(^+\) T cells is reliant on CD28/CD154 costimulation. However, the role of other costimulatory pathways in activation and effector functions of CD4\(^+\) T cells is less clear. Moreover, the impact of growth factors on CD4\(^+\) T cells after CD28/CD154 engagement remains to be clearly defined. CD28 signaling can promote IL-2 production (8), which may, in turn, support clonal expansion of activated T cells, but the precise roles of other growth factors in regulating CD4\(^+\) T cell activation in vivo are not clear. CD8\(^+\) T cells, like CD4\(^+\) T cells, can express CD28 and CD154 (9). However, activation of CD8\(^-\) T cells, at least in certain transplant models, is less dependent on CD28 and CD154 (10–12). Thus, strategies that selectively target CD8\(^+\) T cells often facilitate CD28/CD154 blockade in prolonging allograft survival (11, 13, 14). In other models, however, CD28 and CD154 costimulation appears to be critical in the activation and effector functions of CD8\(^+\) T cells (15, 16), suggesting that the CD28/CD154-independent nature of CD8\(^+\) activation is not absolute in all models. Also, certain alternative costimulatory pathways, such as CD48 or 4-1BB, can preferentially support the activation of CD8\(^+\) T cells (17, 18). Hence, how costimulatory signals and growth factor signals interact and integrate into the activation program of CD4\(^+\) and CD8\(^+\) T cells during the allograft response remains uncertain.

In the present study we took a reductionist approach to critically examine the CD4\(^+\) and CD8\(^+\) T cell subsets and their activation requirement to mediate acute skin allograft rejection using mice deficient for either CD4 or CD8. We found certain key differences between CD4\(^+\) and CD8\(^+\) T cells in their activation requirements to execute skin allograft rejection.

Materials and Methods

**Animals**

Wild-type (wt) C57BL/6 (H-2\(^b\)), B6.CD4 knockout (KO; H-2\(^b\)), B6.CD8 KO (H-2\(^b\)), and DBA/2 (H-2\(^b\)) mice, 8–10 wk old, were purchased from...
The Jackson Laboratory (Bar Harbor, ME). CD28<sup>−/−</sup>CD154<sup>−/−</sup> double-deficient mice were generated by crossing CD28<sup>−/−</sup> mice and CD154<sup>−/−</sup> mice, and their offspring, which were deficient for both CD28 and CD154 genes, were selected by PCR-assisted genotyping and used for the current study. Animal use and care conformed to the guidelines established by the animal care committee at Beth Israel Deaconess Medical Center (Boston, MA).

Reagents

The following Abs used for cell surface staining were purchased from BD Pharmingen (San Diego, CA): CyChrome anti-mouse CD4 (clone RM4-5), CyChrome anti-mouse CD8α (clone 53-6.7), and CyChrome-isotype control Abs.

CTLA-4Ig was a gift from Dr. R. Peach (Bristol-Myers Squibb, Princeton, NJ). Anti-CD154 (MR1, hamster IgG) and anti-OX40 ligand (OX40L; clone RM134L, goat IgG2b) (19) were manufactured from their respective hybridoma lines by BioExpress (West Lebanon, NH). Rat anti-mouse common γ-chain (γc) mAbs (clone 4G3/3E12) were produced and used as we previously reported (20, 21). Rapamycin was provided by Wyeth-Ayerst (Princeton, NJ) and prepared in carboxyethylcellulose for i.p. injections.

Skin transplantation

Full-thickness tail skin grafts (~1 cm²) from donor mice were transplanted onto the thoracic wall of recipient mice. The skin grafts were secured with an adhesive bandage for the initial 5 days. Graft survival was followed by daily visual inspection. Rejection was defined as the complete necrosis and loss of viable skin tissue.

Treatment of skin graft recipients

Treatment of recipient mice with rapamycin consisted of 3 mg/kg/day i.p. on days 0, 1, and 2, followed by treatment every other day for 2 wk as previously reported (22). CTLA-4Ig was given at 0.5 mg i.p. on days 0, 1, and 3, and MR1 was given at 0.5 mg i.p. on days 0, 1, 3, and 6 after skin grafting. Treatment with anti-OX40L mAb consisted of 0.5 mg i.p. on days 0, 2, 4, and 8 after skin transplantation.

Depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells

A previously described protocol was adopted for depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the periphery (23). Mice were given the depleting anti-CD4 mAb (clone GK1.5, rat IgG2b) or the depleting anti-CD8 mAb (clone 2.43, rat IgG1) at 0.2 mg i.p. on days −6, −3, and −1 before skin transplantation, and skin transplantation was performed on day 0. No more depleting mAbs were given thereafter.

Real-time PCR

Real-time PCR was performed using commercially available MasterMix (Applied Biosystems, Foster City, CA). cDNA samples along with primer pairs and specific FAM-labeled fluorescent probes were mixed and amplified in a 25-μl reaction mixture. A predeveloped 18S ribosome RNA primer and probe were used as the internal control. All specific primers and probes used in the study were designed by Primer Express 1.5 software and synthesized by Applied Biosystems. 18S ribosome RNA labeled with VIC dye was purchased from Applied Biosystems. The PCR schema included incubating the samples for 2 min at 50°C to optimize uracil-N-glycosylase activity, followed by 10 min at 95°C, 15 s at 95°C for 40 cycles, and 60 s at 60°C. Quantification of all target genes was analyzed based on a standard comparative threshold cycle (C<sub>T</sub>) method. The average C<sub>T</sub> of each sample and the internal control 18S rRNA were calculated. C<sub>T</sub> values of the target genes were normalized by the control 18S RNA gene, and the final values of target genes were calculated.

CFSE assay

Splenic leukocytes from CD4<sup>+</sup> or CD8<sup>+</sup>-deficient donor mice were prepared and labeled with the vital dye fluorescent CFSE (CFSE-labeled MoBios, Portland, OR) as previously described (24). CFSE-labeled cells (~6–8×10<sup>5</sup>) were then injected via the tail vein into DBA/2 hosts that were irradiated with a Gammancell Exactor (1000 rad; Kanata, Ontario, Canada) shortly before donor cell transfer. Three days later, the host mice were sacrificed, and spleen and peripheral lymph nodes were harvested. The division history of CFSE-labeled donor cells in the host spleen and lymph nodes was examined by flow cytometry based on the dilution profile of the CFSE dye. The large number of cells injected (~6–8×10<sup>5</sup>) and the time frame examined (3 days after cell transfer) preclude homeostatic expansion, and cell proliferation in this model is stimulated primarily by alloantigens (22).

In some experiments the host mice were treated with rapamycin. Rapamycin was given i.p. at 3 mg/kg/day for 3 consecutive days starting with i.v. injection of CFSE-labeled cells. Treatment with anti-γc mAbs consisted of 1 mg daily for 3 days starting at i.v. injection of CFSE-labeled cells. Animals treated with rat IgG (Sigma-Aldrich, St, Louis, MO) were included as controls.

Cell staining and flow cytometry

For analysis of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets, CFSE-labeled cells were recovered from the allogeneic hosts 3 days after adoptive cell transfer. Cells were resuspended in PBS/0.5% BSA (2×10<sup>6</sup>/ml) and stained with CyChrome-conjugated anti-mouse CD4 and CyChrome-conjugated anti-mouse CD8α, respectively, for 10 min on ice, washed in PBS/BSA, and fixed in 1% formaldehyde before analysis.

All samples were analyzed using a FACSort equipped with CellQuest software (BD Biosciences, Mountain View, CA). Data were collected and analyzed by electronically gating on the CD4<sup>+</sup> and CD8<sup>+</sup> populations, respectively. At least 100,000 events were collected for each sample.

Calculation of cell expansion and division frequency

The division frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells at a given time point in the allogeneic hosts was calculated as previously reported (24). Briefly, the division history of donor cells was identified based on their CFSE dilution profiles. The absolute number of cells in each cell division was calculated using the FACS acquisition software CellQuest. The number of precursors that proliferated and gave rise to the absolute number of daughter cells in each cell division was extrapolated using the formula: y<sup>n</sup> (where y is the absolute number of daughter cells in each cell cycle, and n is the number of cell divisions). For example, 16 daughter cells in the third cell division is the progeny of two precursors, each of which have divided three times (i.e., 16/2<sup>3</sup> = 2). The total number of progenies divided by the total number of precursors that have divided represents the magnitude of clonal expansion. The division frequency was then calculated by dividing the total number of precursors by the sum of total CFSE-labeled cells collected.

Histopathology

The skin graft was removed from recipient mice at specified time points after transplantation, fixed in 10% formalin, and embedded in paraffin. Serial tissue sections (5 μm) were prepared and mounted on SuperFrost Plus glass slides (Fisher Scientific, Pittsburg, PA), fixed in methanol, and stained in H&E for histological evaluation.

Results

CD4-deficient mice and CD8-deficient mice promptly reject skin allografts

Although studies using MHC class I/II-deficient mice (23, 25), CD4/CD8 knockout mice (12), or selective CD4<sup>+</sup>/CD8<sup>+</sup> T cell depletion (26) suggest that either CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells can mediate an acute allograft rejection response, studies in certain CD4- and CD8-deficient strains (i.e., BALB/c background, H<sub>2</sub>-<sup>d</sup>) showed that CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells, are essential for
allograft rejection (27). To critically examine the CD4⁺ and CD8⁺ T cell subsets in mediating transplant rejection and their activation requirements to execute such effector function, we transplanted fully MHC-mismatched DBA/2 (H-2b) skin allografts onto B6.CD4KO and B6.CD8KO mice (H-2b), and graft survival was determined and compared with that in wt C57BL/6 (H-2b) mice. As shown in Fig. 1, CD4KO and CD8KO mice promptly rejected the DBA/2 skin allografts. In CD4KO mice, in which rejection is mediated solely by the activation of CD8⁺ T cells, the mean survival time (MST) of the skin allograft was 12 days (n = 8). Similarly, in CD8KO mice, in which rejection is CD4⁺ dependent, the MST of the skin allografts was only 8 days (n = 8). Thus, there was no
marked difference in graft survival compared with the wt controls (MST, 8 days; n = 6). In either group, massive leukocytic infiltration and extensive tissue damage were evident upon histological examination (data not shown). Clearly, either the CD4⁺ or CD8⁺ subset can mediate a vigorous rejection response in this model.

**CD4⁺ and CD8⁺ T cell proliferations in vivo exhibit different sensitivities to growth factor blockade**

Robust proliferation of activated T cells often proceeds acute allograft rejection, and this process is believed to require TCGFs. To quantitatively examine CD4⁺ and CD8⁺ T cell expansion in vivo at a single-cell level without the effect of cross-regulating each other, spleen cells from CD4-deficient mice and CD8-deficient mice were labeled with the vital dye CFSE and injected into lethally irradiated DBA/2 hosts. The division history of donor CD4⁺ and CD8⁺ T cells proliferating in the allogeneic hosts was examined 3 days later (22). As shown in Fig. 2A, CD4⁺ and CD8⁺ T cells proliferated vigorously in vivo, and up to eight cell divisions could be identified. Mathematical calculation revealed that ~590 donor CD4⁺ T cells entered the cell cycle, divided up to eight times, and produced ~13,071 daughter cells. Thus, the mass of alloreactive CD4⁺ T cells expanded >22 times at this time point. Such a magnitude of CD4⁺ T cell proliferation represented a division frequency of ~20.4%. In the CD8⁺ compartment, ~1,450 donor CD8⁺ T cells successfully divided one to eight times and generated ~16,266 daughter cells with a division frequency of 33.1%. Transfer of CFSE-labeled cells into irradiated syngeneic hosts (60–80 × 10⁶ CFSE-labeled cells) did not induce marked T cell proliferation (Fig. 1A), suggesting that T cell proliferation in this model is stimulated primarily by the host alloantigens.

As shown in Fig. 2B, treatment of the host mice with rapamycin, a potent inhibitor of mammalian target of rapamycin (mTOR) activation required for cell cycle progression (28), markedly inhibited the in vivo proliferation of CD8⁺ T cells. Compared with the control group, the most striking effect of rapamycin was the inhibition of late CD8⁺ expansion, especially the seventh division, and this fraction was nearly abolished by rapamycin. The effect of rapamycin on CD4⁺ T cell proliferation was clearly different. Rapamycin had some effect on CD4⁺ T cells in delaying cell cycle entry, but the majority of CFSE-labeled CD4⁺ T cells, once they entered the cell cycle, were capable of dividing many times (more than four times). In fact, the division frequency of CD4⁺ T cells in rapamycin-treated mice (~18%) was comparable to that in untreated controls (~20%).

To ascertain that the different sensitivities of CD4⁺ and CD8⁺ T cells to growth factor blockade is not unique to rapamycin, we treated the DBA/2 hosts with blocking mAbs against γc, a key signaling component shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (4, 5) at the time of cell transfer, and proliferation of donor CD4⁺ and CD8⁺ T cells in the allogeneic hosts was examined simultaneously. Similar to the effect of rapamycin, anti-γc mAbs preferentially inhibited the in vivo proliferation of CD8⁺ T cells, whereas the proliferation of CD4⁺ T cells was not markedly affected by the anti-γc mAbs (Fig. 2C). Clearly, CD4⁺ T cell proliferation in vivo in response to alloantigen stimulation is more resistant to growth factor blockade than the CD8⁺ T cells.

**CD8⁺, but not CD4⁺, T cell-mediated rejection is blocked by rapamycin**

To further examine the role of growth factor signals in CD4⁺ and CD8⁺ T cell-mediated allograft rejection, CD4KO and CD8KO mice were grafted with DBA/2 skin allografts and treated with rapamycin. The treatment protocol consisted of 3 mg/kg/day i.p on days 0, 1, and 2, followed by treatment every other day for 14 days, which has proven efficacy in certain transplant models (22). As shown in Table I, treatment of wt mice with rapamycin only slightly prolonged skin allograft survival (MST, 16 days; n = 8) compared with the untreated controls (MST, 8 days; n = 7). In contrast, long term skin allograft survival was readily achieved in the CD4KO recipients, and all rapamycin-treated CD4KO mice uniformly accepted the skin allograft for >120 days (n = 5). Real-time PCR analysis showed complete inhibition of intragraft expression of CTL markers (perforin, granzyme B, and Fas ligand; data not shown). Surprisingly, the same rapamycin protocol completely failed to prevent skin allograft rejection in CD8KO mice, and all rapamycin-treated CD8KO mice rejected the DBA/2 skin graft with an MST of 20 days (n = 6; Table I). Rapamycin given at a much higher dose (6 mg/kg) also failed to prevent skin allograft rejection in this model (data not shown). Hence, the sensitivities of CD4⁺ and CD8⁺ T cell-mediated rejection to growth factor blockade are strikingly different.

**CD4⁺ T cell-mediated rejection in the absence of CD28/CD154 costimulation is sensitive to rapamycin treatment**

The proliferation of activated CD4⁺ T cells, even with the genetic deficiency of γc (29), suggests that other pathways besides growth factor signaling may also support the effector function of CD4⁺ T cells. To test the possible role of costimulatory pathways in this regard, we transplanted the DBA/2 skin allografts onto CD28/CD154 double knockout (DKO) mice in which both CD28 and CD154, two conventional T cell costimulatory molecules, are genetically knocked out, and graft survival was determined. As shown in Table II, CD28/CD154 DKO mice promptly rejected the DBA/2 skin allografts with an MST of 11 days (n = 6), with only a slight delay compared with the wt controls (MST, 8 days; n = 7).

Depletion of either CD4⁺ T cells or CD8⁺ T cells in the CD28/CD154 DKO mice did not prevent skin allograft rejection, and all CD4⁺-depleted or CD8⁺-depleted DKO mice rejected the DBA/2 skin allografts (MST, 14–17 days; n = 4; Fig. 3A). Analysis at the time of complete graft loss showed a gross depletion of CD4⁺ or CD8⁺ T cells in the DKO recipients, and all rapamycin-treated CD4KO mice uniformly accepted the skin allograft for >120 days (n = 5). Real-time PCR analysis showed complete inhibition of intragraft expression of CTL markers (perforin, granzyme B, and Fas ligand; data not shown). Surprisingly, the same rapamycin protocol completely failed to prevent skin allograft rejection in CD8KO mice, and all rapamycin-treated CD8KO mice rejected the DBA/2 skin graft with an MST of 20 days (n = 6; Table I). Rapamycin given at a much higher dose (6 mg/kg) also failed to prevent skin allograft rejection in this model (data not shown). Hence, the sensitivities of CD4⁺ and CD8⁺ T cell-mediated rejection to growth factor blockade are strikingly different.

### Table I. Effect of rapamycin (RAPA) on skin allograft survival in CD4⁻ and CD8⁻ deficient mice

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Treatment</th>
<th>Skin Graft Survival (days)</th>
<th>n</th>
<th>MST (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>WT C57BL/6</td>
<td>Control</td>
<td>7, 7, 7, 8, 8, 9</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>DBA/2</td>
<td>WT C57BL/6</td>
<td>RAPA</td>
<td>11, 12, 13, 14, 17, 18, 22</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>DBA/2</td>
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<td>14, 16, 18, 22, 22, 23</td>
<td>6</td>
<td>20</td>
</tr>
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</table>

* DBA/2 tail skin was grafted on day 0, and RAPA was given (3 mg/kg/day) on days 0, 1, and 2, followed by treatment every other day for 14 days. Graft survival (days) was determined and is shown.
CD8\(^+\) T cells, respectively (Fig. 3B). Furthermore, treatment of CD4KO mice (i.e., CD8\(^+\) T cell-mediated response) or CD8KO mice (i.e., CD4\(^+\) T cell-mediated response) with CTLA-4Ig and MR1 to block both CD28 and CD154 pathways also failed to block skin allograft rejection, and all skin allografts were rejected within 24 days after transplantation (Table III), suggesting that CD4\(^+\) T cells, like CD8\(^+\) T cells, remain capable of mediating skin allograft rejection in the absence of both CD28 and CD154 molecules.

However, the effect of rapamycin on CD4\(^+\) T cell-mediated rejection in the absence of CD28 and CD154 costimulation is strikingly different. As shown in Fig. 4, treatment of CD8KO mice with rapamycin in combination with CD28 and CD154 blockade, in contrast to rapamycin alone (Table I) or CD28/CD154 blockade alone, induced long term skin allograft survival (MST, >120 days; \(n = 5\)). Furthermore, treatment of CD28/CD154 DKO mice with rapamycin, in contrast to wt controls, induced long term skin allograft survival regardless of CD8\(^+\) depletion (Table II). In fact, 60\% of the DKO recipients treated with rapamycin accepted the skin allografts for >120 days, whereas rapamycin-treated wt control mice rejected DBA/2 skin allografts with an MST of 16 days (\(n = 8\); Table II), suggesting that the absence of CD28/CD154 costimulation renders CD4\(^+\) T cell-mediated rejection sensitive to rapamycin.

Both CD4\(^+\) and CD8\(^+\) T cell-mediated rejections require CD28/CD154 and OX40 costimulation

The ability of rapamycin to prevent skin allograft rejection in CD28/CD154 DKO mice (Table II) as well as in CD4 KO mice (Table I) suggests that effector differentiation of CD28/CD154-deficient CD4\(^+\) T cells and CD8\(^+\) T cells is supported primarily by rapamycin-sensitive growth factor signals. It is unclear whether alternative costimulatory pathways play any role in this regard, or whether activation of such T cells is simply costimulation independent. We recently found that among the known novel costimulatory molecules examined (i.e., inducible costimulator, OX40, 4-1BB, and CD27), OX40 plays a key role in the activation of CD28/CD154-deficient T cells (30), but it remains unclear how OX40 costimulation would affect the CD4\(^+\) or CD8\(^+\) T cell-mediated rejection.

The MST of the skin allograft was >98 days (\(n = 5\)). In contrast, blocking either CD28/CD154 pathways alone or the OX40/OX40L pathway alone only slightly prolonged skin allograft survival (MST, 15–19 days) compared with the untreated controls (MST, 12 days). Similarly, treatment of CD8KO mice with CTLA-4Ig, pathway alone only slightly prolonged skin allograft survival (MST, 15–19 days) compared with the untreated controls (MST, 12 days). Similarly, treatment of CD8KO mice with CTLA-4Ig, pathway alone only slightly prolonged skin allograft survival (MST, 15–19 days) compared with the untreated controls (MST, 12 days). Similarly, treatment of CD8KO mice with CTLA-4Ig,

### Table II. Effect of rapamycin (RAPA) on skin allograft survival in CD28/CD154 DKO mice

<table>
<thead>
<tr>
<th>Donor</th>
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<th>Treatment</th>
<th>Skin Graft Survival (days)</th>
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<th>MST (days)</th>
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<tbody>
<tr>
<td>DBA/2</td>
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<td>Control</td>
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<td>4</td>
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</table>

\(^*\) DBA/2 tail skin was transplanted onto wt C57BL/6 and DKO mice on day 0, and RAPA was given (3 mg/kg daily) for 3 days, followed by treatment every other day for 14 days. In some experiments RAPA was given to a cohort of DKO recipients depleted of CD8\(^+\) T cells and skin graft survival was determined and is shown.

**FIGURE 3.** A. Skin allograft survival in CD4\(^+\) and CD8\(^+\) T cell-depleted CD28/CD154 DKO mice. A depleting anti-CD4 mAb (clone GK1.5) and a depleting anti-CD8 mAb (clone 2.43) were given to a cohort of CD28/CD154 DKO recipients to deplete CD4\(^+\) or CD8\(^+\) T cells before skin transplantation. Skin allograft survival was determined, plotted, and presented. B. Analysis of CD4\(^+\) and CD8\(^+\) T cell depletion in the periphery of CD28/CD154 DKO mice. Spleen cells were prepared from CD28/CD154 DKO mice treated with depleting anti-CD4 or anti-CD8 mAb at the time of complete graft loss. A single-cell suspension was stained with different CyChrome-conjugated anti-CD4 (clone RM4-5) and anti-CD8 mAb (clone 53-6.7) Abs. Cell staining was analyzed by FACS. Cells from untreated mice were used as controls. Representative data from three experiments are shown.
MR1, and anti-OX40L mAb induced long term skin allograft survival (MST, >100 days; n = 8), whereas CD8KO mice treated with anti-OX40L alone or CTLA-4Ig/MR1 alone promptly rejected the skin allografts (Table III). These data suggest that CD4+ and CD8+ T cell-mediated rejection in the presence of CD28/CD154 blockade is supported by OX40 costimulation.

**Discussion**

CD4+ and CD8+ T cells are the two major T cell subsets in the periphery, and differential activation of both T cell subsets probably determines the nature of the rejection response and also the tolerance induction strategies. Thus, understanding precisely the activation requirements for the execution of CD4+ and CD8+ T cell-mediated rejection is critically important in transplantation. In the present study we took a reductionist approach to examine the individual CD4+ and CD8+ T cell subsets in skin allograft rejection and recovered several interesting findings.

A key finding of our study is that the sensitivities of CD4+ and CD8+ T cell-mediated rejection to growth factor blockade are strikingly different. Analysis of CFSE-labeled cells proliferating in the allogeneic hosts showed that either CD4+ or CD8+ T cells can undergo robust proliferation in vivo in response to alloantigen stimulation (Fig. 2). T cell expansion in this model probably reflects their intrinsic features of alloreactivity, as responding T cells undergo robust proliferation in vivo in response to alloantigen stimulation (Fig. 2). T cell expansion in this model probably reflects their intrinsic features of alloreactivity, as responding T cells can be broadly sensitive to mTOR blockade (32).

Another interesting finding is that either CD4+ or CD8+ T cell-mediated rejection is sensitive to costimulatory blockade, and besides conventional CD28/CD154 blockade, blocking the novel CD4+ T cell-mediated rejection sensitive to rapamycin, and long term skin allograft survival can be readily induced under such conditions (Fig. 4). Indeed, activation of Janus kinase 3, a key tyrosine kinase involved in growth factor signaling (34, 35), has been shown to be involved in CD40/CD154 signaling (36). In the present study we showed that blocking CD28/CD154 costimulation or genetic mutation of CD28/CD154 molecules rendered CD4+ T cell-mediated rejection sensitive to rapamycin, and long term skin allograft survival can be readily induced under such conditions (Fig. 4). However, activation of certain costimulatory pathways may have overlapping roles in determining the effector function of CD4+ T cells, and either is sufficient to support the CD4+ T cell-mediated rejection response. These data are consistent with the idea that the proliferation of CD4+ T cells can be growth factor independent, and certain costimulatory pathways are sufficient to drive effector differentiation of activated CD4+ T cells (14, 29, 37).

Another interesting finding is that either CD4+ or CD8+ T cell-mediated rejection is sensitive to costimulatory blockade, and besides conventional CD28/CD154 blockade, blocking the novel OX40 costimulatory pathway is critically important in this regard. Clearly, in either CD4+ or CD8-deficient mice, transient blockade of CD28/CD154/OX40 costimulatory pathways induced long term skin allograft survival, whereas blocking either one of them or either two combinations had minimal effect (Table III). This finding has several important implications. First, despite the unusual pathway of Ag presentation (i.e., direct Ag presentation) during the cell-mediated rejection, as rapamycin preferentially inhibited the in vivo expansion of CD8+ T cells and uniformly produced long term skin allograft survival in CD4-deficient mice (MST, >120 days). Such a rapamycin effect is not due to its selective toxicity to the CD8+ T cells, because blocking the γc, a critical signaling component shared by receptors for all known TCGFs (4, 5), exhibits similar effects on CD8+ T cells. Surprisingly, such exquisite sensitivity to mTOR blockade is not observed for CD4+ T cell-mediated rejection. In fact, the overwhelming majority of CD4+ T cells remain capable of entering the cell cycle regardless of rapamycin treatment or blocking the γc, and prompt skin allograft rejection readily occurs even with rapamycin treatment (Table I). Activated CD4+ T cells are clearly responsive to γc-dependent cytokines in vitro (31), but it is not clear why CD4+ T cells are so resistant to mTOR or γc blockage in vivo. It is possible that other cytokines that are independent of mTOR or γc signaling events may support the effector function of CD4+ T cells in vivo. However, certain evidence suggests that CD28/CD154 costimulatory signals may directly mediate clonal expansion of activated CD4+ T cells. The cytoplasmic tail of CD28 can recruit and activate the phosphotyrosylinositol 3-kinase/Akt pathway (32), a key signaling pathway involved in cell proliferation and cell survival (33). Furthermore, CD28 can directly regulate certain cell cycle regulators, thus promoting cell cycle entry (32). Recently, activation of Janus kinase 3, a key tyrosine kinase involved in growth factor signaling (34, 35), has been shown to be involved in CD40/CD154 signaling (36). In the present study we showed that blocking CD28/CD154 costimulation or genetic mutation of CD28/CD154 molecules rendered CD4+ T cell-mediated rejection sensitive to rapamycin, and long term skin allograft survival can be readily induced under such conditions (Fig. 4). However, activation of certain costimulatory pathways may have overlapping roles in determining the effector function of CD4+ T cells, and either is sufficient to support the CD4+ T cell-mediated rejection response. These data are consistent with the idea that the proliferation of CD4+ T cells can be growth factor independent, and certain costimulatory pathways are sufficient to drive effector differentiation of activated CD4+ T cells (14, 29, 37).

**FIGURE 4.** Effects of CD28/CD154 costimulatory blockade and rapamycin (RAPA) on skin allograft survival in CD8+ T cell-deficient mice. DBA/2 tail skin was grafted onto the CD8+ T cell-deficient mice on day 0. CTLA-4Ig was given i.p. at 0.5 mg on days 0, 1, and 3; MR1 was given at 0.5 mg on days 0, 1, 3, and 6; and anti-OX40L was given i.p. on days 0, 2, 4, and 8 after skin grafting. RAPA was given at 3 mg/kg daily for 3 days, followed by every other day for 14 days.

**Table III. Effect of CD28/CD154/OX40 costimulatory blockade on skin allograft survival in CD4- and CD8-deficient mice**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Treatment</th>
<th>Skin Graft Survival (days)</th>
<th>n</th>
<th>MST (days)</th>
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<td>Control</td>
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<td>Anti-OX40L</td>
<td>10, 12, 18, 20</td>
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<td>15</td>
</tr>
<tr>
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<td>CTLA-4Ig/MR1/anti-OX40L</td>
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<td>5</td>
<td>&gt;98</td>
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<tr>
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<td>Control</td>
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<td>8</td>
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<td>8</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* DBA/2 tail skin was grafted onto CD4- or CD8-deficient mice on day 0. CTLA-4Ig was given (0.5 mg, i.p.) on days 0, 1, and 3; MR1 was given (0.5 mg i.p.) on days 0, 1, 3, and 6; and anti-OX40L was given i.p. on days 0, 2, 4, and 8 after skin transplantation.
allograft response and the high affinity TCR-alloantigen interactions (i.e., a strong signal 1) (38), engagement of costimulatory receptors is absolutely critical to the activation of alloreactive T cells. Apparently, costimulatory pathways involved in this process are redundant, and targeting multiple pathways is required to prevent the rejection response (Table III). Second, OX40 costimulation plays a key role in the activation of both CD4+ and CD8+ T cells in this model, and the costimulatory function of OX40 is independent of CD28 and CD154 signals. Unlike CD28, OX40 is not constitutively expressed on naïve T cells and is expressed only upon T cell activation (7). The expression and costimulatory function of OX40 are not contingent on CD28 and CD154, as OX40-sensitive T cells remain capable of mediating skin allograft rejection in the absence of both CD28 and CD154 (Table III) (30), and blocking the OX40 pathway induces long term skin allograft survival when the conventional CD28/CD154 signals are also blocked (Table III). Third, the alloreactive repertoire is extremely diverse; it is likely that the identities of cells activated upon engagement of different costimulatory pathways are different despite the possibility that either subset can mediate the rejection response. Of particular interest is the finding that OX40 costimulation often supports the generation of memory T cells (39). Thus, OX40-sensitive T cells may be destined to become memory T cells upon alloantigen priming, or such T cells are an endogenous population of memory T cells normally present in the alloreactive pool. The fine differences among diverse T cell subsets in the CD4 and CD8 compartments with respect to their amenable to tolerance induction certainly deserve further investigation.

Our study also suggests that interactions among costimulatory pathways and T cell growth factors during the allograft response are likely to be complex. CD8+ T cell-mediated rejection is sensitive to either CD28/CD154/OX40 costimulatory blockade or growth factor blockade, as transient blockade of CD28/CD154/OX40 pathways or a brief course of rapamycin consistently induced long term skin allograft survival in the CD4-deficient mice (Tables I and III). Thus, it seems likely that a key role of such costimulatory pathways is to promote growth factor production and/or to ensure growth factor responsiveness (e.g., receptor expression and configuration) for the CD8+ T cells. Both components are probably required for effector differentiation of CD8+ T cells, and one cannot compensate for the other during the CD8+ T cell-mediated rejection response. Thus, blocking either CD28/CD154/OX40 pathways or mTOR signaling events prevents CD8+ T cell-mediated rejection. In contrast, CD4+ T cell-mediated rejection has certain features distinct from those of CD8+ T cells. Clearly, CD4+ T cell-mediated rejection is extremely resistant to rapamycin treatment, but such a response can be rendered sensitive to rapamycin when CD28/CD154 costimulatory signals are blockade or genetically absent (Table II and Fig. 4), suggesting that CD28/CD154 costimulatory signals may confer mTOR blockade resistance and may directly stimulate effector differentiation of certain activated CD4+ T cells. It is also possible that the identities of growth factors available to drive T cell proliferation are distinct upon engagement of different costimulatory pathways, or responsiveness to such growth factors is differentially regulated in the CD4 and CD8 compartments. Clearly, more studies are warranted to further unravel this issue.

Thus, differences between CD4+ and CD8+ T cells are more than just MHC class I vs MHC class II restriction as recently reviewed (40), and certain aspects of CD4+ and CD8+ T cell responses are also regulated by different mechanisms. Although the potential interactions between CD4+ and CD8+ T cells in vivo remain to be addressed, the differences identified in the present study between CD4+ and CD8+ T cell-mediated rejection should provide critical insights into a detailed mechanistic understanding of allograft rejection and aid in designing therapeutic strategies for tolerance induction.

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References


