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Cutting Edge: Blockade of the CD28/B7 Costimulatory Pathway Inhibits Intestinal Allograft Rejection Mediated by CD4⁺ But Not CD8⁺ T Cells

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The effect of blocking the CD28/B7 costimulatory pathway on intestinal allograft rejection was examined in mice. Murine CTLA4Ig failed to prevent the rejection of allografts transplanted into wild-type or CD4 knockout (KO) mice but did inhibit allograft rejection by CD8 KO recipients. This effect was associated with decreased intragraft mRNA for IFN-γ and TNF-α and increased mRNA for IL-4 and IL-5. This altered pattern of cytokine production was not observed in allografts from murine CTLA4Ig-treated CD4 KO mice. These data demonstrate that blockade of the CD28/B7 pathway has different effects on intestinal allograft rejection mediated by CD4⁺ and CD8⁺ T cells and suggest that these T cell subsets have different costimulatory requirements in vivo. The results also suggest that the inhibition of CD4⁺ T cell-mediated allograft rejection by CTLA4Ig may be related to down-regulation of Th1 cytokines and/or up-regulation of Th2 cytokines. The Journal of Immunology, 1999, 163: 2358–2362.

Attempts to prevent the rejection of intestinal allografts using this approach have met with mixed results. Although Tarumi et al. reported that CTLA4Ig promoted the long-term survival of intestinal allografts in rats (6), Yin et al. found that CTLA4Ig alone had no effect on intestinal allograft survival (7). The failure of CTLA4Ig to uniformly prevent the rejection of intestinal allografts may be related to different mechanisms responsible for the rejection of intestinal allografts and other allografts. For example, rejection of cardiac allografts in mice is dependent upon CD4⁺ T cells, whereas CD8⁺ T cells are neither necessary nor sufficient (8, 9). In contrast to these findings, we have shown that either CD4⁺ T cells or CD8⁺ T cells are sufficient to cause rejection of intestinal allografts in mice (10). We hypothesized that blockade of the CD28/B7 pathway by murine CTLA4Ig (mCTLA4Ig) may be less effective at preventing the rejection of intestinal allografts due to a differential ability to inhibit CD4⁺ and CD8⁺ T cells in vivo. To test this hypothesis, we compared the effect of mCTLA4Ig on the rejection of intestinal allografts in wild-type (wt), CD4 knockout (KO), and CD8 KO mice. Our data demonstrate that mCTLA4Ig blocked the CD4⁺ T cell-dependent rejection of intestinal allografts in CD8 KO recipients. However, intestinal allograft rejection in wt or CD4 KO recipients was not inhibited by mCTLA4Ig. Supporting the hypothesis that CTLA4Ig is less effective at blocking CD8⁺ T cell-mediated allograft rejection. Our data also demonstrate that the inhibition of CD4⁺ T cell-mediated intestinal allograft rejection by mCTLA4Ig was associated with a decreased expression of Th1 cytokines (IFN-γ and TNF-α) and an increased expression of Th2 cytokines (IL-4 and IL-5).

Materials and Methods

Mice

C57BL/6/J (H-2b), B6C3F1/J (C57BL/6J × C3H/HeJ, H-2ªb), C57BL/6-Cd4 tmlMak (CD4 KO, H-2ª), and C57BL/6-Cd8a tmlMak (CD8 KO, H-2ª) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Reagents

mCTLA4Ig was provided by Mary Collins (Genetics Institute, Boston, MA). Where indicated, recipient mice were treated with 50 μg of mCTLA4Ig administered i.p. every other day for 14 days beginning on the day of transplantation.

Abbreviations used in this paper: mCTLA4Ig, murine CTLA4Ig; wt, wild type; KO, knockout.

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Technique for intestinal transplantation

Intestinal transplantation was performed as described previously (10). After resection of the colon, the donor small bowel was flushed in situ with sterile saline, removed, and stored in sterile saline at 4°C. Intestinal grafts were revascularized by anastomosing the graft portal vein to the recipient inferior vena cava and the graft superior mesenteric artery to the recipient abdominal aorta. The donor jejunum was exteriorized as a stoma, and the graft ileum was anastomosed to the side of the recipient jejunum.

Assessment of intestinal grafts

Samples from the midportion of grafts were fixed in 10% buffered formalin and embedded in paraffin. Sections (3 μm) were stained with hematoxylin-eosin. Acute rejection was scored from 0 to 3 by a blinded pathologist (0, no rejection; 1, scattered apoptotic crypt cells; 2, focal crypt destruction; and 3, mucosal ulceration or sloughing).

Study design

To avoid graft-vs-host disease after intestinal transplantation, a F1 into parent model was used. Allografts procured from B6C3F1/J mice (H-2b k) were transplanted into recipients with a C57BL/6J background (H-2b). Mice that died or developed necrotic stomas during the first 3 days post-transplantation were classified as technical failures and were excluded from analysis.

Semiquantitative RT-PCR

Total RNA was isolated from portions of intestinal grafts frozen in liquid nitrogen using an RNasy Mini Kit (Qiagen, Hilden, Germany). A total of 3–5 μg of total RNA was reverse transcribed using the First Strand cDNA Synthesis kit (Amersham Pharmacia, Piscataway, NJ). PCR amplification was performed using 30-μl reaction mixtures containing RT mixtures: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.125 mM dNTP, 0.025 U/μl Taq Polymerase (Life Technologies, Gaithersburg, MD). The amplification protocol consisted of an initial denaturation at 94°C for 5 min; each cycle of amplification consisted of three steps in which the reaction was denatured at 94°C for 45 s, annealed at the temperature indicated (Table I) for 1 min, and elongated at 72°C for 2 min for a total of 20 or 25 cycles. The elongation stage of the final cycle was extended to 7 min.

For semiquantitative RT-PCR, two rounds of PCR amplification were performed. The starting amount of cDNA used for each sample in the first round of amplification was equalized based on the intensity of GAPDH bands detected after a trial PCR. The first round of PCR amplification was performed using primers for each cytokine and GAPDH. Three tubes (each containing 3 μl of first round PCR products) were used for second round PCR reactions. For each cytokine and GAPDH, one tube was removed after 10, 15, and 20 cycles. PCR products for cytokines and GAPDH were compared by determining the intensity of the bands detected on 2% agarose gel using a Kodak Gel Photography System and Kodak 1D software (version 2.1).

Statistical analysis

Rejection grades were compared using the one-way ANOVA test.

Results and Discussion

CTLA4Ig does not prevent rejection of intestinal allografts by wt mice

To determine the importance of the CD28/B7 pathway for intestinal allograft rejection, wt allograft recipients were treated with mCTLA4Ig. As shown in Fig. 1A and B, mCTLA4Ig did not inhibit the rejection of intestinal allografts. Fig. 1A shows the histologic appearance of representative grafts. Although the syngeneic graft shows no evidence of rejection, the allografts

A

No Treatment

CTLA4Ig_Treated

Syngeneic

Allogeneic

Allogeneic

Allogeneic + mCTLA4Ig

B

FIGURE 1. mCTLA4Ig does not inhibit the rejection of intestinal allografts in wt mice. A demonstrates the histologic appearance on day 14 of representative intestinal allografts transplanted into untreated and mCTLA4Ig-treated wt recipients. B shows the mean rejection grades of the intestinal grafts transplanted into these recipient groups (CTLA4Ig-treated group, n = 8; untreated group, n = 10; syngeneic group, n = 4).

Table I. Amplification primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
<th>Sequence Position</th>
<th>PCR Product Size (bp)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5′-CTGCACCCACCCACGTCTTAG-3′ forward 435 660 57</td>
<td>5′-GTCGAGGATTTGAAAGAT-3′ reverse 1075</td>
<td>295 212 51</td>
<td>487</td>
</tr>
<tr>
<td>IL-2</td>
<td>5′-AGGCCGACAGAATGGAAAGAT-3′ forward 70 362 51</td>
<td>5′-CAGAAGTCCACACAGTTG-3′ reverse 412</td>
<td>362 362 51</td>
<td>412</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5′-ACAATGACCGTACACCTG-3′ forward 366 523 57</td>
<td>5′-TCAAAGCTGCAATACCTATT-3′ reverse 869</td>
<td>70 362 51</td>
<td>362</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′-GGCTCCCCCCTTCATGCTTCT-3′ forward 152 266 55</td>
<td>5′-AACCCCCATTCCCCCTTCACAG-3′ reverse 398</td>
<td>70 362 51</td>
<td>362</td>
</tr>
<tr>
<td>IL-4</td>
<td>5′-TCCACCGGATTGCGACAAAAT-3′ forward 173 197 51</td>
<td>5′-TGAATCCGAGCGTGAAGAA-3′ reverse 350</td>
<td>70 362 51</td>
<td>362</td>
</tr>
<tr>
<td>IL-5</td>
<td>5′-ACAGGAATGAGAGCAGT-3′ forward 70 362 51</td>
<td>5′-GCTGTCCTTCCTCCGACACCTT-3′ reverse 350</td>
<td>70 362 51</td>
<td>362</td>
</tr>
</tbody>
</table>

Annealing temperatures for GAPDH primer were: 59°C. Scores are seen in Table II.
transplanted into either the untreated or mCTLA4Ig-treated wt recipients show severe rejection. As shown in Fig. 1B, there was no difference in the mean rejection grades of allografts transplanted into untreated or mCTLA4Ig-treated wt recipients. These results differ from those reported by Tarumi et al., who reported that CTLA4Ig resulted in long-term survival of intestinal allografts and in the development of donor-specific tolerance in a rat model using low-responder strain combinations (6). This difference may be due at least in part to the different species examined or may be related to the selection of a low-responder strain combination. The latter explanation is consistent with the observation that CTLA4Ig, when administered alone to high-responder rats, failed to inhibit intestinal allograft rejection (7). In this report, however, the combination of CTLA4Ig and an anti-CD4 mAb, unlike either agent alone, did induce long-term intestinal allograft survival. We have shown that an anti-CD4 mAb alone inhibited intestinal rejection in wt mice (10). These data suggest that this mAb, like other anti-CD4 mAbs, might be capable of promoting the development of regulatory T cells. These regulatory cells could in turn inhibit CD8+ T cells and prevent allograft rejection. The requirement for both CTLA4Ig and an anti-CD4 mAb in the rat model may be related to the inadequacy of either alone to induce this type of regulatory population in that model.

**CTLA4Ig prevents rejection of intestinal allografts by CD8 KO but not CD4 KO mice**

Although mCTLA4Ig failed to inhibit the rejection of intestinal allografts by wt recipients, it did prevent the rejection of cardiac allografts in the same strain combination (G.H. and K.A.N., unpublished observations). These data together with the knowledge that CD8+ T cells are sufficient to mediate the rejection of intestinal but not cardiac allografts in mice (9, 10) lead to the hypothesis that the different effects of mCTLA4Ig in these two models may be due to a differential effect on CD4+ or CD8+ T cells. To test this hypothesis, CD8 KO and CD4 KO mice were treated with mCTLA4Ig after intestinal transplantation. As shown in Fig. 2, A and B, mCTLA4Ig inhibited the rejection of intestinal allografts only in CD8 KO recipients (p < 0.001 vs untreated CD8 KO mice). This effect persisted long-term, as the mean rejection grade of intestinal allografts from mCTLA4Ig-treated CD8 KO mice examined between days 60 and 120 was 1.2 ± 0.7 (p < 0.01 vs untreated CD8 KO mice examined on day 14).

The prevention of intestinal allograft rejection by mCTLA4Ig in CD8 KO but not CD4 KO recipients provides evidence that the costimulatory requirements of CD4+ and CD8+ T cells are different in this model. The costimulatory requirements of CD8+ T cells in vivo have also been investigated in other models. It has been reported that TCR transgenic CD8+ T cells can function in an allogeneic tumor model in the absence of a CD28/B7 signal (11). However, another group reported that a B7 costimulatory signal augmented CD8+ T cell responses to tumor cells (12). In a model of graft-vs-host disease and in a viral model, anti-B7 mAbs have been shown to inhibit the development of CD8+ effector cells (13, 14). Taken together, these data and our data suggest that although costimulation through the CD28/B7 pathway may augment the function of CD8+ T cells in some systems, this pathway is not always necessary to develop effective CD8+ T cell responses.

**Inhibition of intestinal allograft rejection by CTLA4Ig in CD8 KO mice is associated with a decrease in Th1 cytokines and an increase in Th2 cytokines**

In several transplant models, a deviation from Th1 to Th2 cytokines has been associated with long-term allograft acceptance (reviewed in Refs. 15 and 16). Semiquantitative RT-PCR was used to compare cytokine production within intestinal allografts transplanted into untreated or mCTLA4Ig-treated wt, CD4 KO, or CD8 KO mice. As shown in Fig. 3, no mRNA for IL-2, IFN-γ, or IL-4 was detected in syngeneic grafts, and only a small amount of mRNA for TNF-α was detected. mRNA for IL-2, IFN-γ, TNF-α, and IL-4 was readily detected in allografts transplanted into untreated wt, CD4 KO, or CD8 KO mice. mCTLA4Ig had no effect on the relative amounts of mRNA for IL-2 detected in treated groups. However, the inhibition of allograft rejection by mCTLA4Ig in CD8 KO mice was associated with a decreased production of IFN-γ and TNF-α and an increased production of IL-4 and IL-5 (Fig. 3, data not shown for IL-5). This pattern was not observed in rejecting allografts that had been transplanted into CD4 KO mice treated with mCTLA4Ig. In allografts surviving long-term in mCTLA4Ig-treated CD8 KO mice, little or no mRNA for IL-2, IFN-γ, or TNF-α was detected, whereas a significant amount of mRNA for IL-4 was detected (Fig. 3). These data are similar to a report that CTLA4Ig inhibited the production of Th1 cytokines but spared Th2 cytokines in a rat kidney transplant model (17). Our data are compatible with a model in which mCTLA4Ig prevents CD4+ T cell-mediated rejection of intestinal allografts by inhibiting the production of Th1 cytokines and/or
promoting the production of Th2 cytokines. Alternatively, the changes in cytokine production noted after treatment with CTLA4Ig may be a consequence of allograft acceptance and not the cause. However, the observation that the shift from Th1 to Th2 cytokines was observed at early (day 14) as well as late (day 90) timepoints supports a model in which the shift in cytokine profiles promotes allograft acceptance.

In summary, our data demonstrate that CD4\(^+\) T cells are dependent upon the CD28/B7 pathway for the rejection of intestinal allografts. CD8\(^+\) T cells, on the other hand, are capable of rejecting intestinal allografts independent of this costimulatory signal. This suggests that CD8\(^+\) T cells are either independent of all costimulatory signals or are dependent upon different costimulatory molecules than are CD4\(^+\) T cells.
References


