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Cutting Edge: Membrane Lymphotoxin Regulates CD8+ T Cell-Mediated Intestinal Allograft Rejection

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Blocking the CD28/B7 and/or CD154/CD40 costimulatory pathways promotes long-term allograft survival in many transplant models where CD4+ T cells are necessary for rejection. When CD8+ T cells are sufficient to mediate rejection, these approaches fail, resulting in costimulation blockade-resistant rejection. To address this problem we examined the role of lymphotoxin-related molecules in CD8+ T cell-mediated rejection of murine intestinal allografts. Targeting membrane lymphotoxin by means of a fusion protein, mAb, or genetic mutation inhibited rejection of intestinal allografts by CD8+ T cells. This effect was associated with decreased monokine induced by IFN-γ (Mig) and secondary lymphoid chemokine (SLC) gene expression within allografts and spleens respectively. Blocking membrane lymphotoxin did not inhibit rejection mediated by CD4+ T cells. Combining disruption of membrane lymphotoxin and treatment with CTLA4-Ig inhibited rejection in wild-type mice. These data demonstrate that membrane lymphotoxin is an important regulatory molecule for intestinal allografts. Targeting membrane lymphotoxin by means of mig expressed by T cells and activated B cells to lymphotoxins, showing inducible expression, competing with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), which is expressed by immature dendritic cells (DC), resulting in enhanced T cell proliferation and cytokine production, suggesting that HVEM/LIGHT interactions provide a costimulatory signal for T cells (12). The binding of Mig expressed by T cells and activated B cells to lymphotoxin β receptor (LTβR) expressed on stromal cells and cells of monocytic origin has been shown to be critical for the development of lymphoid organs (13) and for the regulation of chemokine production (14). Disruption of these molecules has been reported to impair the immune response to viruses and tumors (15–19).

The purpose of the current study was to determine whether manipulation of these lymphotoxin-related molecules could be used to inhibit costimulation blockade-resistant, CD8+ T cell-mediated allograft rejection. To test this hypothesis we used a heterotopic murine intestinal transplant model. This model offers the advantage that, unlike the murine cardiac transplant model, either CD4+ or CD8+ T cells can mediate rejection (20). However, because this model lacks physical characteristics amenable to serial, noninvasive monitoring, and because costimulation blockade fails to prevent rejection in a number of transplant models. This phenomenon has been termed costimulation blockade-resistant rejection. Initial examples include the failure of CTLA4-Ig to inhibit the rejection of intestinal allografts and the inability of an anti-CD154 mAb to inhibit skin graft rejection (7, 8). The failure of CD28 and CD154 blockade to prolong allograft survival has been shown to correlate with resistance of CD8+ T cells to the blockade of these molecules (7–11). Thus, while costimulatory blockade effectively inhibits CD4+ T cell-mediated rejection, similar approaches to control CD8+ T cell-mediated rejection have not been described. Lymphotoxin-related proteins, which belong to the TNF/TNFR superfamilies, are also important regulators of immune responses and as such are promising targets for modulating recipient responses to transplanted organs. Members of the lymphotoxin-related family of proteins expressed by T cells include herpes virus entry mediator (HVEM)5 and membrane lymphotoxin (mLT). HVEM binds a TNF-like molecule homologous to lymphotoxins, showing inducible expression, competing with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), which is expressed by immature dendritic cells (DC), resulting in enhanced T cell proliferation and cytokine production, suggesting that HVEM/LIGHT interactions provide a costimulatory signal for T cells (12). The binding of Mig expressed by T cells and activated B cells to lymphotoxin β receptor (LTβR) expressed on stromal cells and cells of monocytic origin has been shown to be critical for the development of lymphoid organs (13) and for the regulation of chemokine production (14). Disruption of these molecules has been reported to impair the immune response to viruses and tumors (15–19).

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5 Abbreviations used in this paper: HVEM, herpes virus entry mediator; LTβR, lymphotoxin β receptor; mLT, membrane lymphotoxin; LIGHT, a TNF-like molecule homologous to lymphotoxins, showing inducible expression, competing with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes; DC, dendritic cell; SLC, secondary lymphoid chemokine; MRG, mean rejection grade; Mig, monokine induced by IFN-γ; GVHD, graft-vs-host disease.

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recipient survival is not dependent upon the survival of the heterotopic graft, the assessment of transplanted intestines was based upon their histologic appearance.

**Materials and Methods**

**Mice**

C57BL/6 (H-2^b_), C57BL/6 × C3H/Hdj (B6C3Fl/J, H-2^bk_), and C57BL/6-Cd4tm10ak (Cd4^-/-, H-2^b_) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD). LTx^-/- mice, back-crossed six generations onto the C57BL/6 background, have been previously described (21).

**Intestinal transplantation and histologic graft assessment**

Intestinal transplantation was performed as described (20). Intestine grafts were revascularized by anastomosing the portal vein to the recipient inferior mesenteric vein, the superior mesenteric artery to the recipient infrarenal aorta. The jejunum was exteriorized as a stoma and the ileum was anastomosed to the side of the recipient jejunum. Specimens for histologic assessment were fixed in 10% buffered formalin and embedded in paraffin. H&E-stained 3-µm sections were evaluated by a pathologist in a “blinded” fashion. Rejection was graded according to the following definitions: 0, no rejection; 1, scattered apoptotic crypt cells; 2, focal crypt destruction; and 3, mucosal ulceration with or without transmural necrosis.

**Study design**

To avoid graft-vs-host disease (GVHD), a F1 into parent transplant model was used. Allografts were procured from B6C3Fl/J mice (H-2^bk_). The genetic background of all recipient mice was C57BL/6/J (H-2^b_). Technical failures, defined as mice that died within the first 3 days, were excluded from analysis.

**Fusion proteins and mAb**

LTIIR-Ig is comprised of the LTIIR extracellular domain attached to the C\_\_\_\_2 and C\_\_\_\_3 domains of human IgG1 (13). Recipient mice were treated with 100 µg administered i.p. on days 0 and 7. Mice in control groups were treated with human IgG. The fusion protein mCTLA4-Ig was provided by M. Collins (Genetics Institute, Cambridge, MA). Recipient mice were treated with 50 µg of mCTLA4-Ig administered i.p. every other day for 14 days beginning on the day of transplantation. B6F1-BF2, a mAb that is specific for the β-chain of mIg, was provided by J. Browning (Biogen, Cambridge, MA). This mAb was administered i.p. at a dose of 100 µg on days 0, 3, and 7.

**RT-PCR**

Total RNA was isolated from intestinal grafts frozen by liquid nitrogen using a RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples were treated with RNase-free DNase (Amersham Pharmacia Biotech, Piscataway, NJ). Total RNA (3-5 µg) was reverse transcribed using the First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech). The mRNA encoding the H\_2 and C\_\_\_\_3 domains of human IgG1 (13). Recipient mice were treated with 100 µg administered i.p. on days 0 and 7. Mice in control groups were treated with human IgG. The fusion protein mCTLA4-Ig was provided by M. Collins (Genetics Institute, Cambridge, MA). Recipient mice were treated with 50 µg of mCTLA4-Ig administered i.p. every other day for 14 days beginning on the day of transplantation. B6F1-BF2, a mAb that is specific for the β-chain of mIg, was provided by J. Browning (Biogen, Cambridge, MA). This mAb was administered i.p. at a dose of 100 µg on days 0, 3, and 7.

**Results and Discussion**

As an initial approach toward determining the role of lympho-toxin-related molecules in allograft rejection, intestinal allografts were transplanted into LTx^-/- recipients. Although there was a trend toward a decrease in the severity of rejection in LTx^-/- recipients (p = 0.07 vs wild-type controls), all recipients did develop rejection (Fig. 1). Recent reports describing an association between disruption of mlT and defects in host immunity have linked this effect to impaired function of CD8\_+ T cells (15, 17). Thus, we hypothesized that the rejection observed in LTx^-/- mice was mediated by CD4\_+ T cells. We have previously shown that CD4\_+ T cell-mediated rejection can be blocked by CTLA4-Ig (7).

To distinguish between these possibilities, we treated CD4^-/- intestinal allograft recipients with LTIIR-Ig that binds mIg and LIGHT but not LTx. We have previously shown that in CD4^-/- mice rejection is dependent upon CD8\_+ T cells (20). Treatment with LTIIR-Ig significantly inhibited CD8\_+ T cell-mediated rejection at day 14 (Fig. 2, p < 0.01). This protective effect persisted at day 28, although three of the four treated mice had developed moderate rejection (mean rejection grade (MRG) 1.5 ± 1.0, n = 4, p = 0.04 vs day 14 CD4^-/- control group). These data demonstrate a role for either mIg or LIGHT in the process of intestinal allograft rejection and imply that the defect in CD8\_+ T cell-mediated rejection in LTx^-/- mice is at least in part due to the disruption of mIg. The failure of LTIIR-Ig to promote long-term allograft survival in this model could be due to incomplete blockade of mIg and LIGHT or the

![FIGURE 1.](http://www.jimmunol.org/)

**FIGURE 1.** CTLA4-Ig inhibits intestinal allograft rejection in LTx^-/- recipients. Recipient mice were treated as described with CTLA4-Ig. On day 14 following transplantation, grafts were assessed histologically. Day 14 syngeneic grafts showed no evidence of rejection (rejection score, all grafts, 0). Each ✶ represents the score for an individual recipient. The heavy lines (→) represent the mean score for the indicated group.
ability of other effector mechanisms to mediate rejection independent of mLT and LIGHT.

Unlike the results obtained using LTα−/− mice, the results obtained using LTβR-Ig cannot be attributed to an alteration in the structure of secondary lymphoid organs or loss of expression of LTα. However, in addition to binding mLT, LTβR-Ig also binds to LIGHT (24). The interaction of LIGHT with HVEM has been shown to deliver a signal that costimulates T cells (12, 19). Inhibition of the HVEM/LIGHT interaction by LTβR-Ig has been reported to inhibit tumor growth, prevent the development of GVHD, and impair the development of alloreactive CTL (18, 19). Thus, the inhibition of allograft rejection associated with LTβR-Ig could either be a consequence of its ability to bind mLT or its ability to bind LIGHT, thereby blocking the HVEM/LIGHT costimulatory pathway.

To distinguish between the effects of blocking mLT and LIGHT on the rejection of intestinal allografts by CD8+ T cells, we treated CD4−/− recipient mice with BRF6-BF2, a mAb specific for the β-chain of the mLT complex. Blockade of mLT using this anti-LTβ mAb significantly inhibited the rejection of intestinal allografts by CD4−/− mice (Fig. 2, p < 0.01). These data confirm those obtained using LTβR-Ig and directly demonstrate that mLT contributes to CD8+ T cell-mediated allograft rejection. Interestingly, the anti-LTβ mAb had little effect on intestinal allograft rejection mediated by CD4+ T cells in CD8−/− recipients (MRG 2.0 ± 0, n = 3). These data describe an important new role for mLT in allograft rejection and identify a novel strategy to inhibit costimulation blockade-resistant rejection. However, it should be noted that these data do not exclude a potential contribution of LIGHT to this process.

Mechanisms by which biological agents inhibit rejection can be grouped into those that deplete alloreactive T cells, those that prevent complete T cell activation and induce anergy by blocking costimulatory signals, those that alter T cell differentiation, those that impair T cell migration, and those that induce the development of regulatory cells. Of these possible mechanisms, the engagement of HVEM by LIGHT costimulates T cells (12) and the engagement of mLT by LTβR augments chemokine production (14), which in turn regulates cell migration. We postulated that one or both of these mechanisms contributed to the protective effect of disrupting lymphotoxin-related molecules on intestinal allograft rejection and undertook studies to test this hypothesis. Characteristically, agents that block T cell costimulatory molecules prevent complete T cell activation and consequently inhibit Ag-driven T cell proliferation. Unlike CLTA4-Ig, LTβR-Ig did not inhibit the proliferation of naïve T cells to alloantigens in vitro over a broad range of concentrations nor did it inhibit the proliferation of CD4+ or CD8+ T cells in vivo in a GVHD model (data not shown). These data suggest that LTβR-Ig may inhibit rejection by mechanisms other than the prevention of T cell costimulation and activation.

Not having observed an effect of LTβR-Ig on T cell costimulation, its effect on chemokine production was examined. Given the demonstrated role of Mig in allograft rejection (25), the effect of LTβR-Ig and the anti-LTβ mAb on Mig gene expression was determined. Treatment of either wild-type or CD4−/− recipient mice with LTβR-Ig significantly reduced Mig gene expression within intestinal allografts (Fig. 3, A and B). The anti-LTβ mAb also significantly reduced Mig gene expression in CD4−/− recipients (Fig. 3B). These data, together with the knowledge that migration of immune cells is regulated by concentration gradients of chemoattractant molecules such as Mig, suggest that LTβR-Ig and anti-LTβ mAb-induced alterations in chemokine production within intestinal allografts may inhibit rejection by impairing the migration of leukocytes to the allograft. Although our data do not directly test this hypothesis, the importance of this potential mechanism warrants future investigation.

In addition to affecting the production of Mig, which regulates the migration of immune cells to sites of inflammation in peripheral tissues, mLT has been reported to affect the production of “lymphoid chemokines” such as SLC (14). The engagement of CCR7 by SLC has been reported to play an important role in bringing DC and T cells together in secondary lymphoid...
FIGURE 4. Treatment with LTβR-Ig or an anti-LTβ mAb is associated with decreased SLC gene expression in the spleens of wild-type and CD4−/− mice bearing intestinal allografts. mRNA was isolated from the spleens of recipient mice treated with LTβR-Ig or the anti-LTβ mAb BBF6-BF2. Each * represents the value corresponding to a single sample. The heavy lines (—) denote the mean value. Data are displayed as the fold change in the ratio of SLC:GAPDH for the experimental groups relative to the syngeneic group.

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