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

Zhong Guo,2* Jun Wang,2* Lingzhong Meng,† Qiang Wu,‡ Oliver Kim,‡ John Hart,‡ Gang He,§ Ping Zhou,§ J. Richard Thistlethwaite, Jr.,† Maria-Luisa Alegre,§ Yang-Xin Fu,3‡ and Kenneth A. Newell3,4*.

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 CD8+ T cells mediating rejection and suggest a strategy to avoid costimulation blockade-resistant rejection. The Journal of Immunology, 2001, 167: 4796–4800.

In addition to the TCR, several molecules expressed by T cells function as important regulators of immune responses (1). A subset of these molecules, costimulatory molecules, are promising targets for manipulation in many disease states (2). In many experimental transplant models blockade of the CD28/B7 or CD154/CD40 costimulatory pathways has been shown to inhibit the rejection of allogeneic and xenogeneic grafts in rodents and primates (3–6). Despite the frequent success of this strategy, it has been increasingly recognized that costimulatory 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 mLT 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 molecular interactions 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

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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-2b), C57BL/6 × C3H/HdJ (B6C3F1/J, H-2b/Kb), and C57BL/6-Cd4tmSak (CD4+/-; H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD). LTα−/− 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 inframesenteric 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 B6C3F1/J mice (H-2b/Kb). The genetic background of all recipient mice was C57BL/6J (H-2b). Technical failures, defined as mice that died within the first 3 days, were excluded from analysis.

Fusion proteins and mAb

LTβR-Ig is comprised of the LTβR extracellular domain attached to the Cγ2 and Cγ3 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. BBF6-BF2, a mAb that is specific for the β-chain of mLT, 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 GAPDH, Mig, and SLC were detected by real-time RT-PCR using the ABI Prism 7700-sequence detection system (Applied Biosystems, Foster City, CA) as described (11). The primer and probe sequences were designed using the software program Primer Express Version 1.0 (Applied Biosystems). Sequences used to detect GAPDH, Mig, and SLC are as follows: GAPDH forward primer, 5′-TTCACCAACTACTGGAGAAGGC-3′; reverse primer, 5′-GGCATGGACTGTTGCTATGA-3′; probe, 5′-TGCTACCTCTCACCACCAACTACTGGAGAAGGC-3′; Mig forward primer, 5′-GCCATGAAAATGCCTGTTCT-3′; reverse primer, 5′-GGTTCCTCGAATCTCCACAC-3′; probe, 5′-TTCCTTTTGGGCGATCATCTCCGTGA-3′; SLC forward primer, 5′-AGACTCAGAGCCCAACGACCA-3′; reverse primer, 5′-GTGGATAGGACGCAAGGAGG-3′; and probe, 5′-CCACCTCTAGCTGGCTCCCTGTC-3′.

Reactions were performed using 20-μl reaction volumes and the TaqMan Universal PCR master mixture (Applied Biosystems). PCR conditions were 50°C for 2 min, 95°C for 10 min. Each amplification was 15 s at 95°C and 1 min at 60°C for 40 cycles. PCR results were analyzed using the relative standard curve method.

Statistical analysis

Rejection grades were compared using the Kruskal-Wallis test for samples from multiple groups and the Mann-Whitney U test for samples from two groups. Continuous variables were compared using the unpaired t test with Welch correction. Calculations were performed using InStat version 2 (GraphPad, San Diego, CA). Values of p < 0.05 were considered significant.

Results and Discussion

As an initial approach toward determining the role of lymphocticin-related molecules in allograft rejection, intestinal allografts were transplanted into LTα−/− recipients. Although there was a trend toward a decrease in the severity of rejection in LTα−/− 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 LTα−/− mice was mediated by CD4+ T cells. We have previously shown that CD4+ T cell-mediated rejection can be blocked by CTLA4-Ig (7). Therefore, to test the hypothesis, LTα−/− recipient mice were treated with CTLA4-Ig. Rejection was completely inhibited when LTα−/− mice were treated with CTLA4-Ig, whereas CTLA4-Ig had no effect on rejection in wild-type recipients (Fig. 1). These data suggest that LTα-related molecules contribute significantly to the process of allograft rejection and that this effect is predominantly on CD8+ T cells. Several mechanisms could explain this effect. Secreted lymphokin LTα, which is absent in LTα−/− mice, may contribute to intestinal allograft rejection. Similarly, mlT, which is comprised of LTα and LTβ, is also absent in LTα−/− mice and may play a role in allograft rejection. Alternatively, the disruption of splenic architecture and the paucity of lymph nodes associated with the LTα−/− phenotype (22) may be responsible for the impaired immune response to intestinal allografts. This is consistent with the observation that splenectomized al/j mice, which lack lymph nodes and splenic tissue, fail to reject heart allografts (23).

To distinguish between these possibilities, we treated CD4−/− intestinal allograft recipients with LTβR-Ig that binds mLT and LIGHT but not LTα. We have previously shown that in CD4−/− mice rejection is dependent upon CD8+ T cells (20). Treatment with LTβR-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 mLT or LIGHT in the process of intestinal allograft rejection and imply that the defect in CD8+ T cell-mediated rejection in LTα−/− mice is at least in part due to the disruption of mLT. The failure of LTβR-Ig to promote long-term allograft survival in this model could be due to incomplete blockade of mLT and LIGHT or the
Mechanisms by which biological agents inhibit rejection can be grouped into those that deplete alloreactive T cells, those that impair T cell migration, and those that induce blocking costimulatory signals, those that alter T cell differentiation. However, it should be noted that these data do not exclude a potential contribution of LIGHT to this process. 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<sup>−/−</sup> 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<sup>−/−</sup> recipients (Fig. 3B). These data, together with the knowledge that migration of immune cells is regulated by concentration gradients of chemokine 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 structures. Consequently, inhibition of CCR7 by SLC has been reported to play an important role in inhibiting 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.

To distinguish between the effects of blocking mLT and LIGHT on the rejection of intestinal allografts by CD8<sup>+</sup> T cells, we treated CD4<sup>−/−</sup> recipient mice with BBF6-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<sup>−/−</sup> mice (Fig. 2, p < 0.01). These data confirm those obtained using LTβR-Ig and directly demonstrate that mLT contributes to CD8<sup>+</sup> T cell-mediated allograft rejection. Interestingly, the anti-LTβ mAb had little effect on intestinal allograft rejection mediated by CD4<sup>+</sup> T cells in CD8<sup>−/−</sup> 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 lymphotxin-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 naive T cells to alloantigens in vitro over a broad range of concentrations nor did it inhibit the proliferation of CD4<sup>+</sup> or CD8<sup>+</sup> 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.

FIGURE 3. Blockade of the mLT pathway inhibits Mig gene expression in intestinal allografts from wild-type (A) and CD4<sup>−/−</sup> (B) recipients. mRNA was isolated from intestinal allografts of recipient mice treated with LTβR-Ig or the anti-LTβ mAb BBF6-BF2. Each symbol represents the value corresponding to a single sample. The heavy lines (—) denote the mean value. Data are shown as the fold change in the ratio of transcripts encoding Mig and GAPDH in the indicated experimental group relative to the syngeneic group.
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 BB6-F62. Each bar 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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