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Blockade of Lymphotoxin Signaling Inhibits the Clinical Expression of Murine Graft-versus-Host Skin Disease

Qiang Wu,* Yang-Xin Fu,† and Richard D. Sontheimer‡

Adhesion molecules are essential for the recruitment of T cells into the skin during the development of graft-vs-host skin disease (GVHSD). However, the mechanisms responsible for the regulation of expression of cutaneous adhesion molecules in this setting are still poorly understood. In this study we blocked lymphotoxin (LT) signaling in a murine model of minor histocompatibility Ag system mismatch GVHSD by using an LTβ receptor-Ig fusion protein (LTβR-Ig). The recipient mice treated with control human Ig developed clinically apparent, severe skin lesions. However, none of the mice treated with LTβR-Ig developed clinical skin disease. The expression of ICAM-1 in cutaneous tissue was also much lower in mice treated with LTβR-Ig than in mice treated with human Ig. Thus, the inhibition of LT signaling via LTβR-Ig treatment appears to be capable of markedly ameliorating the development of GVHSD, possibly by inhibiting the expression of adhesion molecules. The Journal of Immunology, 2004, 172: 1630–1636.

E arly/acute murine graft-vs-host skin disease (GVHSD) can serve as an experimental model for the human lichenoid tissue reaction, a histopathological pattern that is common to human skin diseases such as lupus erythematosus, dermatomyositis, lichen planus, as well as the early/acute phase of human GVHSD (1). In prior studies, our laboratory examined a murine early GVHSD model resulting from minor histocompatibility Ag system (mHA) mismatch (2). Using this model, we previously demonstrated that the infiltration of T cells into the skin is essential for the epidermal basal cell injury pattern that is present in early murine GVHSD lesions (2). Other studies have also implicated activated T cells as the primary effector cell type in the lichenoid tissue reaction (1, 3).

Adhesion molecule-ligand interactions are essential for the regulation of T cell migration into cutaneous tissues (4–8). Some studies have shown that the expression of adhesion molecules is significantly up-regulated in the injured skin induced by GVHD (6, 7). However, the molecular mechanism(s) responsible for the enhanced expression of adhesion molecules on dermal microvessels and epidermal keratinocytes in this setting is poorly understood.

TNF-α, a member of the TNF superfamily, plays a crucial enhancing role in the expression of adhesion molecules on dermal microvessels during the development of lichenoid cutaneous lesions (5, 8). However, blockade of the TNF pathway with anti-TNF Abs has been unable to completely prevent the expression of GVHSD, suggesting that other inflammatory cytokines could be involved in the pathogenesis of lichenoid cutaneous lesions.

LT, another member of the TNF superfamily, is expressed as a soluble homotrimer (LTα3) or as a membrane-bound heterotrimer (LTα1β2; mLT) on activated lymphocytes (9, 10). LTα3 interacts with and signals through TNF receptors I and II and displays similar biological functions as TNF-α in the inflammatory activity (9, 10). However, mLT binds to and signals through the LTβ receptor (LTβR). The interaction between mLT and its receptor is essential for the development of lymphoid tissues and lymphocyte compartmentalization in the spleen as well as for the migration of dendritic cells into lymphoid tissues via controlling the expression of chemokines and adhesion molecules (9–12). Blockade of the mLT-LTβR interaction in nonobese diabetic mice not only effectively prevents the onset of diabetes at the initial phase, but also inhibits the development of diabetes at the stage of insulitis (13). We speculate that LT may be required to up-regulate chemokines and/or adhesion molecules to create the local microenvironment that attracts autoreactive T cells.

We, therefore, questioned whether mLT signaling might play a role in the clinical expression of GVHSD. To answer this question, in the present study mice receiving mHA mismatch bone marrow (BM) and spleen cells were treated with LTβR-Ig. We found that the blockade of mLT signaling by LTβR-Ig treatment could completely inhibit the development of clinically evident murine GVHSD skin lesions. This effect could be mediated by the down-regulation of chemokines and adhesion molecules necessary for T cell homing to the skin.

Materials and Methods

Mice

Female C57BL/6J (B6, H-2b) and LP/J (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the animal care facility at University of Iowa. Both murine strains have multiple minor
histocompatibility locus differences. For this study all mice were female, and donor animals were 7–12 wk of age; recipients were 6–8 wk of age.

**Reagents**

LTβR-Ig was generated according to the method described previously (11, 13). The following anti-mouse Abs were used for immunohistochemistry: purified anti-CD4 (rat Ig2a, clone H129.19), purified anti-CD8 (rat Ig2a, clone 53-6.7), and purified hamster anti-ICAM-1 (Armenian hamster IgG, clone 3E2). All Abs were obtained from BD Pharmingen (San Diego, CA).

**GVHD induction**

The murine GVHSD employed was previously described by our laboratory in detail (2). In brief, BM cells were obtained by flushing the marrow cavities of the femurs and tibiae of donor mice with PBS. A single-cell suspension of donor BM and spleen cells was filtered through sterile nylon mesh and resuspended in PBS. Recipient B6 mice were irradiated with 10 Gy from a 60Co source. Six hours later, the mice were injected i.v. via the retrobulbar venous plexus with 4 × 10^6 splenocytes and 2 × 10^6 BM cells from LP/J or B6 mice in 0.2 ml of PBS. In some experiments recipient mice were injected i.v. with 75 μg of LTβR-Ig or control human Ig in 200 μl of PBS on days 0 and 7 after BM and spleen cell transplantation.

**Evaluation of GVHD disease**

**Body weight.** B6 recipient animals in all groups were weighed weekly throughout the study. A body weight loss of >1 g of initial weight was considered significant. Results were shown by average body weight change (grams).

**Gross skin disease.** B6 recipient animals were observed weekly for the development of clinical skin lesions. Mice first developed clinically evident skin lesions 28 days after transplantation; photographs of representative, clinically evident skin changes were obtained at various time points thereafter.

**Histopathology of skin disease.** Posterior neck skin biopsies were obtained on days 14, 28, and 40 after transfusion and were preserved in methanol, followed by embedding in paraffin. Tissue sections (7 μm) were prepared and stained with H&E. A numerical scoring system was used to grade the patterns of the observed histopathological changes. The scoring system consisted of five levels (from 0 to 4) based on the degree of infiltration of mononuclear cells and thickening of the epidermis and dermis. The levels of scoring included: 0, no signs of infiltration; 1, low density mononuclear cell infiltration in dermis/epidermis; 2, high density of mononuclear cell infiltration in dermis/epidermis; 3, widening/thickening of epidermis; and 4, widening/thickening of the dermis and decrease in number of hair follicles. These histopathological patterns generally reflected the severity of the associated clinical changes observed.

**Demonstration of epidermal keratinocyte apoptosis**

TUNEL staining was conducted according to the manufacturer’s instructions (Roche, Indianapolis, IN) on skin sections using an in situ cell death detection kit employing a fluorescence readout signal. Briefly, posterior neck skin biopsies were embedded in OCT compound (Miles-Yeda, Elkhart, IN), and frozen at -70°C. Frozen sections (7 μm) were prepared and fixed in 4% paraformaldehyde. The sections were incubated with proteinase K (20 μg/ml) for 30 min at 37°C and then incubated with 0.1% Triton X-100 (in 0.1% sodium citrate) for 2 min on ice. After washing in PBS, the sections were incubated with TUNEL reaction mixture for 60 min at 37°C. Sections were then observed under a fluorescence microscope. The number of TUNEL-positive cells in epidermis per linear millimeter was evaluated using ImageJ software (Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD).

**Immunohistochemistry or immunofluorescence**

Posterior neck skin biopsies were fixed in IHC ZinC fixative (BD PharMingen) and embedded in paraffin for immunohistochemistry or immunofluorescence. Methanol-treated sections (7 μm) were stained with H&E by standard methods for evaluation by light microscopy. For T cell demonstrations, 7-μm IHC ZinC-fixed sections were sequentially incubated with 1) rat anti-CD4 or anti-CD8 at a dilution of 1/100, 2) biotinylated mouse anti-rat IgM at a dilution of 1/200, or 3) FITC-conjugated anti-biotin clone BN-34 at a dilution of 1/150 (Sigma-Aldrich, St. Louis, MO). For ICAM-1 demonstration, sections were incubated with 1) hamster anti-ICAM-1 at a dilution of 1/120, 2) biotinylated mouse anti-hamster IgM mixture at a dilution of 1/200, or 3) alkaline phosphatase-conjugated streptavidin. HRP-mediated color development was performed using the 3,3'-diaminobenzidine substrate kit (BD Pharmingen). The numbers of ICAM-1-positive capillaries per square millimeter in cutaneous tissue and of ICAM-1-positive keratinocytes per linear millimeter were evaluated using ImageJ software. In addition, the intensity of the positive capillaries was evaluated using a grading scale of three levels based on the staining intensity: +, weak staining; ++, medium staining; and ++++, strong staining.

**Statistical analysis**

All data were expressed as the mean and SD. The statistical significance of difference between means was determined using two-tailed Student’s t tests. Differences were considered significant at p < 0.05.

**Results**

**Skin lesions expressed in the LP/J>B6 GVHD murine model**

The mHA mismatch LP/J>B6 GVHS model that was employed in the present study has been previously described (2). In this model, recipient B6 mice develop clinical and histopathological evidence of progressive cutaneous GVHD disease (Figs. 1 and 2A). Recipient mice developed the earliest clinical skin lesions, including scruffiness and hair loss over the posterior aspect of the neck, on day 28 after transfusion. Skin biopsy at that time revealed severe mononuclear cell infiltration and increased overall skin thickness (Fig. 2, D and G). By day 40 after transfusion, variable combinations of alopecia, blistering, erosion, crusting, and hyperkeratosis were observed initially, starting on the posterior aspect of the neck and then extending gradually to the face and abdomen (Fig. 1A). Skin biopsy on day 40 after transfusion displayed severe mononuclear cell infiltration, compressed and atrophic hair follicles, and increased overall skin thickness (Fig. 1, B and C). B6>B6 syngeneic control mice did not develop clinically evident skin lesions.

**FIGURE 1.** Skin lesions resulting from LP/J>B6 mHA mismatch. Lethally irradiated B6 recipients were transplanted with LP/J donor BM and spleen cells. A. By day 40 after transfusion, the majority of recipient mice showed clinical skin lesions, including alopecia and scales over posterior neck and face. B and C. Biopsies of the posterior neck skin on day 40 after transfusion were obtained and fixed in methanol. Sections (7 μm) were stained with H&E. Original magnification: B, ×200; and C, ×600.
FIGURE 2. LTβR-Ig treatment effectively blocks the appearance of skin lesions. Lethally irradiated B6 recipients were transplanted with B6 BM and spleen cells (syngeneic controls) or LP/J donor BM and spleen cells (experimental). On days 0 and 7, experimental recipients (n = 9) transplanted with LP/J donor BM and spleen cells were given i.v. injections of 75 μg of LTβR-Ig in 200 μl of PBS or 75 μg of human Ig in 200 μl of PBS (n = 11). Syngeneic recipients (n = 6) were injected i.v. with 200 μl of PBS. A, Approximately 55% of the LP/J→B6 and human Ig-treated mice showed clinical skin lesions, and administration of LTβR-Ig could block the development of such skin lesions. B, LP/J→B6 and human Ig-treated mice exhibited clinically obvious, severe skin lesions. In contrast, LP/J→B6 and LTβR-Ig-treated animals showed no visible skin changes, nor did B6→B6 syngeneic controls. C–E and F–H, Biopsies of the posterior neck skin on day 28 after transfusion from B6→B6 controls (C and F) and LP/J→B6 and human Ig-treated mice (D and
LTβR-Ig treatment inhibits cutaneous GVHD and weight loss

Recent studies, revealed that LT signaling plays an important role in the pathogenesis of autoimmune diabetes and experimental autoimmune myasthenia (13–15). Blockade of LT signaling could prevent CD8-positive T cell-mediatedintestinal allograft rejection (16). Thus, we hypothesized that the LT-LTβR pathway might be involved in the development of GVHSD.

To test this hypothesis, B6 recipient mice were treated with 75 μg of LTβR-Ig on days 0 and 10 after LP/J BM and spleen cell transfusion. By 40 days after transfusion, 55% of control mice treated with human Ig had developed clinically obvious, severe skin lesions consisting of varying elements of blistering, erosion, crusting, hyperkeratosis, and alopecia on the face, ears, neck, and abdomen (Figs. 1A and 2, A and B). However, recipient B6 mice treated with LTβR-Ig and syngeneic B6>B6 control mice had not developed clinical skin lesions at this same time point (Fig. 2, A and B). Compared with B6>B6 mice (Fig. 2, C, F, and I), posterior neck skin biopsies from human Ig-treated control animals revealed an ~200% increase in epidermal thickness and an ~70% increase in dermal thickness due largely to prominent mononuclear cell infiltration on day 28 after transfusion (Fig. 2, D, G, and J). Skin biopsies of mice treated with LTβR-Ig at the same point in time demonstrated only low levels of mononuclear cell infiltration and normal skin thickness (Fig. 2, E, H, and J). The skin pathology score was much higher in control mice treated with human Ig than in B6>B6 mice, and there was no significant difference between mice treated with LTβR-Ig and B6>B6 mice (Fig. 2J).

Although recipient mice in all groups displayed transient body weight loss by days 7–14 due to irradiation toxicity, only mice treated with human Ig showed significant weight loss resulting from GVHD on day 49 after transfusion. In contrast, mice treated with LTβR-Ig and B6>B6 control mice returned to normal body weight after undergoing transient weight loss (Fig. 2K). Similar findings were obtained in two additional replicate experiments. These data suggest that LTβR-Ig treatment can effectively block the clinical expression of murine GVHSD.

LTβR-Ig treatment inhibits T cell homing to skin

Our laboratory has previously demonstrated that T lymphocytes are the primary cellular effectors of epidermal basal cell injury that occurs in the LP/J>B6 model of early murine GVHD skin lesions (2). To determine whether LTβR-Ig treatment could inhibit the infiltration of T cells into the skin in this model, immunofluorescence analysis was conducted on posterior neck skin biopsy sections on day 14 after transplantation in all groups. At the time of biopsy, severe T cell infiltration was detected in the dermis and epidermis in human Ig-treated animals, but no significant difference were detected in the numbers of CD4+ and CD8+ T cells (Fig. 3, A and B). In contrast, very few T cells were detected in the skin of the LTβR-Ig treated animals (Fig. 3, C and D). These data suggest that LTβR-Ig treatment can prevent the infiltration of T cells into skin, suggesting a role for LT signaling in the regulation of T cell recruitment that could be important to the development of GVHD skin lesions.

LTβR-Ig treatment inhibits epidermal keratinocyte apoptosis

Keratinocyte apoptosis can be induced by autoreactive T cells and local inflammatory cytokines (8, 17–19). The presence of apoptotic keratinocytes is a useful early diagnostic criterion for GVHD. Fig. 3 indicates that blockage of mLT signaling can dramatically inhibit T cell recruitment into the skin. We therefore sought to determine whether LTβR-Ig treatment could prevent keratinocyte apoptosis by inhibiting T cell infiltration into skin.

Apoptotic keratinocytes were identified in situ by the TUNEL technique on sections from skin biopsies taken on day 14 after transplantation. More apoptotic cells (green) were present in epidermis and hair follicles of the skin from human Ig-treated control animals than in LTβR-Ig-treated animals (p = 0.007; Fig. 4). These data suggest that mLT signaling might be involved in the induction of keratinocyte apoptosis in part by regulating the recruitment of skin-homing T cells into skin.

LTβR-Ig treatment prevents the expression of adhesion molecules

Enhanced expression of adhesion molecules on the surface of dermal endothelial cells is crucial for autoreactive T cell attachment and migration through the endothelial barrier into cutaneous tissues during the development of autoimmune skin diseases (4–7). LT signaling has been shown to regulate the migration of lymphocytes and dendritic cells via the regulation of adhesion molecule expression in secondary lymphoid organs and inflamed tissues (12, 13, 20, 21). In the present study we have demonstrated that the blockade of the LT-LTβR pathway can markedly decrease the extravasation of T cells into cutaneous tissue. To address a mechanism that might be responsible for this effect, we next examined the expression of adhesion molecules during the development of GVHSD disease in this model.

ICAM-1 was examined by immunohistochemistry on sections from skin biopsies taken on days 14 and 28 after transplantation.

G) and LP/J>B6 and LTβR-Ig-treated mice (E and H). Skin biopsies were fixed in methanol, and 7-μm sections were stained with H&E. Original magnification: C–E, ×100; and F–H, ×600. I. Measurement of dermis and epidermal thickness in these H&E stained sections. Panel J shows the results of the histopathological scoring system described in Materials and Methods. K. Changes in body weight of the animals in the various experimental and control groups over time. All results indicated in Fig. 2 were representative of three independent experiments.
Discussion

In the present study we have demonstrated that lethally irradiated B6 mice receiving LP/J BM and spleen cells develop clinically obvious skin lesions with a histopathology consistent with that of early/acute GVHD. Moreover, there was a significant loss of body weight appearing 10 days after the recipient mice first developed clinically obvious skin lesions. These data suggest that the earliest and predominant target of attack in the mHA mismatch murine GVHD model employed in this study is cutaneous tissue.

Interestingly, the appearance of such skin lesions could be completely blocked by the administration of LTβR-Ig. Such treatment appeared to inhibit the extravasation of T cells into and downregulate ICAM-1 expression within cutaneous tissue. The administration of LTβR-Ig has also been shown by others (22) to inhibit the expression of GVHD, as reflected by an increase in survival...
and a reversal of weight loss via blocking the activation of donor effector T cells. However, neither that study nor our present one has fully excluded the possibility that LTβR-Ig treatment might have also decreased donor cell engraftment and thereby moderated the intensity of the resulting GVHD reaction. This issue will be addressed in future studies in our laboratory.

Local primary inflammatory cytokines such as TNF-α and IL-1 can play important roles in the induction of keratinocyte destruction via directly damaging local tissues and attracting inflammatory cells (5, 8, 17, 18, 23). Within the skin, these cytokines are thought to be produced predominately by keratinocytes and activated T cells (8, 17, 18). LTα3, defined as having similar biological activity as TNF-α, is also found to be expressed on activated keratinocytes and T cells in skin tissue, suggesting that the interaction of LTα3 singling may also be involved in the induction of keratinocyte destruction (24). The prevention of GVHD by LTβR-Ig treatment described in the present study might involve the inhibition of inflammatory cytokines such as LTα3 and TNF-α produced by both keratinocytes and T cells via the blockade of the LT signaling pathway. It will be interesting to examine the LT family member-mediated inflammatory cytokine levels and distribution in this model.

It has been demonstrated that T cells produce epidermal basal keratinocyte injury by cell-mediated cytotoxicity and the production of inflammatory cytokines during the pathogenesis of human disorders that produce a lichenoid tissue reaction such as GVHD, lupus erythematosus, dermatomyositis, and lichen planus (1–3). The impairment of T cell recruitment to the skin could prevent the development of such skin inflammation (25). Our previous work has shown that blockade of LT-mediated autologous T cell infiltration into the pancreas could inhibit the onset of diabetes mellitus in NOD mice (13). The results described in this study further demonstrate that the blockade of LT signaling can effectively inhibit the infiltration of skin-homing T cells into cutaneous tissues, suggesting that LT-mediated recruitment of T cells into skin might be at least partially responsible for the development of GVHD and possibly other lichenoid tissue reaction skin disorders.

T cells programmed to home to cutaneous tissue undergoing immune-mediated inflammation must undergo several critical steps, including attachment to the endothelium and migration through the endothelial barrier. The interactions between adhesion molecules and their ligands are critical steps for the attachment of T cells to the endothelium of cutaneous microvessels. Therefore, better understanding the mechanisms regulating the expression of adhesion molecules is highly relevant to immunotherapy for human skin diseases displaying the lichenoid tissue reaction. ICAM-1 expression has been found to be increased in GVHD, and blocking ICAM-1 expression can ameliorate the development of GVHD (25, 26).

Our results in the present study suggest that blockade of the LT-LTβR signaling pathway can down-regulate the expression of ICAM-1 on endothelial cells in the dermis. Regulation by members of the TNF superfamily of the expression of adhesion molecules has been well established (8, 27, 28). Both LTα3 and TNF-α can up-regulate the expression of adhesion molecules such as ICAM-1 in vitro (8). This adhesion molecule also plays an important role in T cell homing to the skin. The mLT signaling pathway has been shown to be involved in the expression of adhesion molecules in secondary lymphoid organs (20, 21). Inhibition of this signaling pathway by administration of both LTβR-Ig and anti-LTβ Ab can efficiently block the expression of mucosal cell adhesion molecule-1 in the spleen (20, 21). We have previously shown that LTβR-Ig treatment can decrease the homing of dendritic cells to the spleen (11). Therefore, the down-regulation of adhesion molecule expression by LTβR-Ig treatment might be responsible for the inhibition of T cell infiltration into cutaneous tissue. Some work has shown that the expression of ICAM-1 on keratinocytes is increased in the lesional skin of GVHD (6, 7, 29, 30). This high level of ICAM-1 expression is thought to facilitate T cell-mediated target destruction by the attachment of T cells to keratinocytes (7, 29, 30). Interestingly, in the present study we found that the level of ICAM-1 expression on keratinocytes was significantly lower in transplanted mice treated with human Ig than in transplanted mice treated with LTβR-Ig. The down-regulation of ICAM-1 expression on keratinocytes resulting from LTβR-Ig treatment might be very important in protecting keratinocytes from T cell-mediated destruction. Taken together, our experiment suggests that LT-mediated adhesion molecule expression may be important in the development of GVHD.

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