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Controlling herpetic stromal keratitis by modulating lymphotoxin-alpha-mediated inflammatory pathways

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Abstract

Herpes simplex virus 1 infection of the eye can result in stromal keratitis, a chronic immunoinflammatory lesion that is a significant cause of human blindness. A key to controlling the severity of lesions is to identify cellular and molecular events responsible for tissue damage. This report evaluates the role of lymphotoxin- α , a proinflammatory cytokine that could be involved during stromal keratitis. We demonstrate that after infection, both lymphotoxin- α and lymphotoxin- β transcripts are detectable at high levels 48 hours postinfection, suggesting roles for the secreted homotrimer lymphotoxin- β and the membrane-bound lymphotoxin- α 1 2 heterotrimer in stromal keratitis. Using a corneal stromal fibroblast cell line, lymphotoxin- β and lymphotoxin- α 1 2 were found to have proinflammatory roles by stimulating production of chemokines. Treatment of mice with a depleting anti-lymphotoxin- α mAb during the clinical phase of the disease significantly attenuated stromal keratitis lesions. In treated mice, expression of proinflammatory molecules and chemokines was reduced, as were numbers of cornea-infiltrating proinflammatory cells, particularly Th1 cells. The protective effect of anti-lymphotoxin- α mAb was highly reduced with a mutant version of the mAb that lacks Fc receptor binding activity, indicating that depletion of lymphotoxin-expressing cells was mainly responsible for efficacy, with LT- β contributing minimally to inflammation. These data demonstrate that lymphotoxin-expressing cells, such as Th1 cells, mediate stromal keratitis.

Keywords

HSV-1; SK; Th1; lymphotoxin α ; CD4⁺ T cells

1. Introduction

Ocular infection with herpes simplex virus type 1 (HSV-1) can result in a chronic immunoinflammatory lesion in the corneal stroma, which is a significant cause of human vision loss [1, 2]. The pathogenesis of stromal keratitis (SK) involves numerous events, but

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studies indicate that lesions are mainly orchestrated by CD4⁺ T cells. These CD4⁺ T cells recognize virus-derived peptides or even altered self-proteins that are unmasked once the cornea is damaged [3-5]. Therefore, SK lesions largely represent immunopathological reactions and these are managed by prolonged treatment with antiviral and anti-inflammatory drugs [6, 7]. Since there is no currently available treatment that removes latent virus from infected persons, periodic new lesion episodes are to be expected [3, 8, 9]. Current therapies can be problematic, but there is hope that identifying components of lesion pathogenesis and targeting them for therapy could represent a more acceptable form of therapy. One approach could be to eliminate or inhibit the activated proinflammatory CD4⁺ T cells that are mainly responsible for orchestrating SK lesions [10-14].

Lymphotoxin-alpha (LT- α) is a member of the TNF superfamily. It can be found as a soluble homotrimer, known as LT- α_3 , which binds TNF receptors and has been shown to have a proinflammatory role in different animal models of inflammatory diseases [15]. LT- α can also be complexed with LT- β as a heterotrimer, known as LT- $\alpha_1\beta_2$, which binds to its cognate receptor LT- β and is expressed on the cell surface of some lymphocyte populations, including activated CD4⁺ Th subsets such as Th1 and Th17 cells [16, 17]. Targeting LT- α with a mAb was shown to be efficacious in some murine models of autoimmune diseases that involve the inflammatory effects of T cells [16, 17]. Since there are parallels in pathogenic mechanisms in some autoimmune diseases and SK, we speculated that the administration of anti-LT- α mAb could modulate disease in a mouse model of SK.

In the present report we demonstrate that in the initial inflammatory response to HSV-1 infection LT- α is upregulated in the cornea. We also show that the soluble version of LT- α_3 has a proinflammatory role *in vitro* in a corneal cell line. When mice with early lesions of SK were treated with anti-LT- α mAb, lesions were significantly diminished and inflammatory cells and their products were markedly reduced compared to control Ab treated animals. Even though a slight decrease in lesion severity and infiltration of inflammatory cells and presence of inflammatory molecules was observed when using a mutant Ab that lacked Fc receptor binding activity, this decrease was not significant, in contrast with the one found in wild-type Ab treated animals. This indicated that the protective effect involved depletion of LT- α expressing cells. Since activated Th1 cells are one of the predominant pathogenic cells expressing LT- α , our data confirms the role of these cells in mediating SK, but also demonstrates that secreted LT- α_3 has a minimal role in driving disease. Additionally, since this mAb treatment was effective to limit the severity of SK when initiated during the clinical phase, the strategy may be worth exploring in the natural disease situation.

2. Materials and Methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facilities. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Tennessee (Public Health Service Assurance number 63-R-0105). HSV-1 eye infection was performed under deep anesthesia (avertin), and all efforts were made to minimize animal suffering.

2.2. Mice, virus, and cell lines

For the experiments, C57BL/6NHsd female 5 to 6 weeks old were used from Harlan Sprague Dawley Laboratories. All manipulations were done in a laminar flow hood. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-1 RE was used in all procedures. Virus was grown and titrated on Vero cells (American Type Culture Collection no. CCL81) using standard protocols. The virus was stored in aliquots at -80°C until use. MK/T-1 cell line (immortalized keratocytes from C57BL/6 mouse corneal stroma) was kindly gifted by Dr. Reza Dana (Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, MA).

2.3. Corneal HSV-1 infection and clinical observations

Corneal infections of C57BL/6 mice were done under deep anesthesia induced by intra-peritoneal injection in tribromoethanol (avertin) as previously described [18]. Corneas were scarified with a 27-gauge needle, and a 3- μl drop containing the specific viral dose (1×10^4 PFU) was applied to the eye. Eyes were examined on different days postinfection (p.i.) with a slit-lamp biomicroscope (Kowa Company, Nagoya, Japan) measuring the progression of SK lesion severity and angiogenesis of individual mice. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing keratitis [19].

2.4. Treatment

Mice were treated subcutaneously (SC) with LT- -specific mAb (anti-LT-) (6mg per kg body weight), LT- .Fc-MT (anti-LTMT) (6mg per kg body weight), CTLA-4 fusion protein (CTLA-4.Ig) (6mg per kg body weight), or isotype control ragweed-specific mouse IgG2a antibody (6mg per kg body weight) 3 times per week, starting on day 6 p.i. [17].

2.5. Histopathology

Eyes from isotype and treated mice were extirpated on day 15 p.i. and snap frozen in OCT compound (Miles; Elkart, IN, USA). Six-micron-thick sections were cut and air dried in a desiccation box. Staining was performed with hematoxylin and eosin (Richard Allen Scientific; Kalamazoo, MI, USA).

2.6. Flow cytometry

Single cell suspensions were prepared from cornea and cervical DLNs of mice at different time points p.i. Corneas were excised, pooled group wise, and digested with 60 U/ml Liberase (Roche Diagnostics; Indianapolis, IN, USA) for 35 min at 37°C in a humidified atmosphere of 5% CO_2 . After incubation, the corneas were disrupted by grinding with a syringe plunger on a cell strainer, and a single-cell suspension was made in complete RPMI 1640 medium.

The single cell suspensions obtained from corneas and DLNs were stained for cell surface molecules. All steps were performed at 4°C . A total of 1×10^6 cells were first blocked with an unconjugated anti-CD32/CD16 mAb for 30 min in FACS buffer. After washing with FACS buffer, CD4-PerCP (clone RM4.5, BD Biosciences, San Jose, CA, USA) mAb was added for 30 min on ice. Neutrophils were defined as $\text{CD45}^+\text{Ly6G}^+\text{CD11b}^+$ using CD45-allophycocyanin (clone 30-F11, BD Biosciences), CD11b-PerCP (clone MI/70, BD Biosciences) and Ly6G-FITC (clone 1A8, BD Biosciences). Finally, the cells were washed three times and re-suspended in 1% *para*-formaldehyde. The stained samples were acquired with a FACS Calibur (BD Biosciences; San Jose, CA, USA), and the data were analyzed

using FlowJo software. For corneas, total cell numbers were calculated by acquiring the totality of the sample and taking into consideration total number of corneas in the sample.

To enumerate the number of IFN- γ -producing CD4⁺ T cells, intracellular cytokine staining was performed as previously described [13]. In brief, 10⁶ freshly isolated lymph node and corneal cells were cultured in U-bottom 96-well plates. For *in vitro* induced cultures, cells were left unstimulated or stimulated with anti-CD3 (3 μ g/ml) and anti-CD28 (1 μ g/ml) for 5 h in the presence of brefeldin A (10 μ g/ml). Subsequently, cell surface staining was performed, followed by intracellular cytokine staining with anti-IFN- γ -FITC (clone XMG1.2, BD Biosciences), using a Cytotfix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer's recommendations. The fixed cells were resuspended in 1% paraformaldehyde prior to data acquisition.

2.7. MK/T-1 cell assay

Immortalized corneal stromal fibroblast (MK/T-1) cells were stimulated *in vitro* with different concentrations of rmLT-3 (R&D Systems; Minneapolis, MN, USA) in DMEM supplemented with 5% FBS for 6 h at 37°C in 5% CO₂. The effect of LT-3-specific mAb and anti-LTMT in blocking rmLT-3 was confirmed by addition of the Abs to the wells at 0.1 μ M. Cells were collected for gene expression analysis.

2.8. Reverse transcriptase PCR (RT-PCR)

Cultured MK/T-1 cells were lysed, and total mRNA was extracted using RNeasy Mini Kit (Qiagen; Valencia, CA, USA). RT-PCR was performed to detect the presence of mRNA transcripts of various molecules (LT-3, LT-R, TNFR1, TNFR2). cDNAs were amplified by PCR and were resolved on 1% agarose gel. The PCR primers used were the following:

LT-R, F 5'-CCAGGACAAGGAATACTACGAG-3' and R 5'-GGACACAGTTGACGTCAGTATC-3';

TNFR1, F 5'-GAGAAGAGGGATAGCTTGTGTC-3' and R 5'-GGTGTCTGAGTCTCCTTACAG-3';

TNFR2, F 5'-CCAAGGACACTCTACGTATCTC-3' and R 5'-CTACTGCATCCTGGGATTTCTC-3'

2.9. Real-time PCR (Q-RT-PCR)

RNA was extracted from cells and tissue using TRIzol LS reagent (Invitrogen; Carlsbad, CA, USA). Total cDNA was made with 500 ng of RNA using oligo(dT) primer. Q-RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with an iQ5 RT-PCR detection system (Bio Rad, Hercules, CA, USA) using 5 μ l cDNA for 40 cycles. The expression levels of different molecules were normalized to β -actin using the threshold cycle method calculation. For corneas, relative expression between mock-infected samples and isotype or treated samples from day 15 p.i. were calculated using the 2^{-Ct} formula: $Ct = Ct_{\text{sample}} - Ct_{\text{reference}}$. Here Ct is the change in cycling threshold between the gene of interest and the "housekeeping" gene β -actin, where Ct_{sample} was the Ct value for any treated or isotype samples from day 15 p.i. normalized to the β -actin gene, and $Ct_{\text{reference}}$ was the Ct value for the mock-infected samples (scratched and infected only with PBS) also normalized to β -actin. Each of the samples was run in duplicate to determine sample reproducibility, and a mean Ct value for each duplicate measurement was calculated. For *MK/T-1 cells*, relative expression between control (non-exposed) samples and samples exposed to LT-3 with or without anti-LT-3 and anti-LTMT, or exposed to LT-1/2 were calculated using the 2^{-Ct} formula: $Ct =$

$Ct_{\text{sample}} - Ct_{\text{reference}}$. Here, Ct is the change in cycling threshold between the gene of interest and the “housekeeping” gene β -actin, where Ct_{sample} was the Ct value for any sample exposed to LT- α 3 with or without anti-LT- α and anti-LTMT, or exposed to LT- α 1 2 normalized to the β -actin gene, and $Ct_{\text{reference}}$ was the Ct value for the control samples also normalized to β -actin. Each of the samples was run in duplicate to determine sample reproducibility, and a mean Ct value for each duplicate measurement was calculated.

The PCR primers used were the following:

β -actin, F 5'-CCTTCTTGGGTATGGAATCCTG-3' and R 5'-GGCATAGAGGTCTTTACGGATG-3'; IL-1, F 5'-GAAATGCCACCTTTTGACAG-3' and R 5'-CAAGGCCACAGGTATTTTGT-3'; IFN- γ , F 5'-GGATGCATTCATGAGTATTGC-3' and R 5'-GCTTCCTGAGGCTGGATTC-3'; CXCL-9, F 5'-CAAGCCCCAATTGCAACAAA-3' and R 5'-TCCGGATCTAGGCAGGTTTGA-3'; CXCL-10, F 5'-TGCTGGGTCTGAAGTGGGACT-3' and R 5'-AAGCTTCCCCTATGGCCCTCA-3'; MCP-1/CCL2, F 5'-TGGCTCAGCCAGATGCAGT-3' and R 5'-TTGGGATCATCTTGCTGGTG-3'; KC/CXCL1, F 5'-GTGTTGCCCTCAGGGCC-3' and R 5'-GCCTCGCGACCATTCTTG-3';

2.10. Statistical analysis

Most of the analyses for determining the level of significance were performed using one-way ANOVA test. Values $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ were considered significant. Results are expressed as means $\pm$ SEM. For some experiments, as mentioned in the figure legends, an unpaired two-tailed Student t test was applied.

3. Results

3.1. Changes in LT- α and LT- β as a consequence of HSV-1 infection

To investigate the potential role of LT- α in mediating tissue damage during SK, mice were ocularly infected with HSV and then expression levels of both LT- α and LT- β were measured in pools of corneal extracts at various time points p.i. Expression levels of both LT- α (Fig. 1A) and LT- β (Fig. 1B) were readily detectable in infected corneas with a first peak on day 2 p.i. (average 300-fold and 150-fold increase compared to scratched but uninfected controls, respectively). However, expression of both LT- α and LT- β was rapidly down-regulated, as by day 3 mRNA expression levels for both molecules were at control levels. The initial burst of LT- α and LT- β transcriptional activity was followed by a second wave, with marked increases in mRNA levels in the infected group detectable in day 15 samples (LT- α with an average of 40 fold and LT- β with an average of 120 fold). In summary, expression levels of LT- α and LT- β were increased after HSV-1 infection. Both peaks correlated with the infiltration of proinflammatory cells that we see after HSV-1 infection of the cornea [4], and that have been previously described as populations that express LT- α [17].

3.2. LT- α 3 acts as a proinflammatory cytokine in vitro

LT- α can be found as a soluble homotrimer, LT- α 3, and can also be complexed with LT- β as a membrane-bound heterotrimer, LT- α 1 2. To determine whether LT- α 3 acts as a proinflammatory mediator in the cornea, we used the corneal stromal fibroblast cell line MK/T-1. Culture of MK/T-1 cells with recombinant LT- α 3 resulted in significant induction of transcriptional activity of CXCL-10 and KC (Fig. 2A), chemokines involved in the migration of pathogenic cells into the cornea [4]. A mAb specific for LT- α (anti-LT- α) has been shown to bind to both LT- α 3 and LT- α 1 2, but only fully blocks the interaction

between LT-3 and TNFR and not between LT-1/2 and LT-R [17]. Addition of anti-LT-3 to MK/T-1 cells stimulated with LT-3 completely inhibited the secretion of CXCL-10 and KC, as did an Fc-effectorless mutant of the anti-LT-3 mAb which has been shown to have similar binding/blocking properties as the wild-type mAb [17]. MK/T-1 cells stimulated with LT-1/2 were similarly induced to express CXCL-10 and KC (Fig. 2B), but inhibition assays were not possible due to the inability of anti-LT-3 mAb to fully block LT-1/2/LT-R interaction. Because LT-3 and LT-1/2 have a proinflammatory effect on a cell line that is derived from keratocytes of C67BL/6 mice, we can hypothesize that LT-3 and LT-1/2 could act *in vivo* to induce chemokines that further recruit inflammatory cells to the infected cornea.

3.3. LT- α -specific mAb decreases SK induced immunopathology and inhibits cellular infiltration in infected corneas

To evaluate whether LT-3 has a role in mediating SK immunopathology, the anti-LT-3 and anti-LTMT mAbs were used to treat mice in a herpetic SK model. Mice infected with HSV were treated 3 times a week, starting on day 6 p.i. when lesions start to become evident. As seen in Fig. 3, treatment with anti-LT-3 significantly decreased the severity of SK lesions when compared to the isotype control Ab group. SK scores (Fig. 3A) were significantly lower in the anti-LT-3 group (2.6 ± 0.2 vs 3.8 ± 0.3 in the isotype treated animals, $p = 0.01$), eyes exhibited visible reductions in lesion severity (Fig. 3B), and histology showed decreased cellular infiltration and inflammation in the cornea (Fig. 3C). The protective effects of anti-LT-3 were similar to those conferred by CTLA-4.Ig treatment, previously shown to impair the onset and severity of SK [20], and which was used as positive control for assessing T cell contribution to SK in these experiments. In contrast to anti-LT-3 treatment, anti-LTMT did not have an effect on SK outcome, with lesions having similar severity as those in the isotype control group.

In Fig. 2 we demonstrated that anti-LT-3 as well as anti-LTMT could bind to LT-3 and block its activity *in vitro*. Moreover, anti-LTMT is an Fc-effectorless mutant of wild-type anti-LT-3, and is thus unable to mediate antibody-dependent cellular cytotoxicity [17, 21], the efficacy of anti-LT-3 treatment appears to be dependent on depletion of LT-3-expressing cells. To determine whether treatment with anti-LT-3 reduces pathogenic cell numbers in the mouse SK model, pools of corneas were collected on day 15 p.i. from anti-LT-3, anti-LTMT and isotype treated animals and processed for recovery of inflammatory cells for enumeration. CD4⁺ T cells are primary drivers of SK, and anti-LT-3 treatment significantly reduced the frequency of CD4⁺ T cells in the cornea and CD4⁺ T cell numbers on an absolute basis compared to isotype control, having similar effects as CTLA-4.Ig (Fig. 4A). In contrast, anti-LTMT treated animals did not show significant differences of CD4⁺ T cell frequencies or numbers, indicating that most the effects of anti-LT-3 were probably mediated through Fc-dependent mechanisms specifically directed at LT-3-expressing cells.

Within the CD4⁺ T cell population, Th1 cells are the primary orchestrators of the inflammatory reaction [4]. To determine if Th1 cells were selectively affected by anti-LT-3 treatment, cells were isolated from corneal tissues and draining lymph nodes on day 15 p.i., following treatment during the clinical phase of disease. Using intracellular cytokine staining for IFN- γ to identify Th1 cells, anti-LT-3 treatment was found to significantly reduce the frequency and absolute number of Th1 cells in the cornea (Fig. 4B) as well as draining lymph node (Fig. 4C), again having a comparable effect as CTLA-4.Ig treatment. As observed with bulk CD4⁺ T cells, anti-LTMT treatment showed no significant effect on Th1 cells when compared to the isotype treated group in neither corneas nor DLNs, although a trend towards reduction of cell frequencies and numbers was noticed.

3.4. LT- α -specific mAb treatment reduces proinflammatory responses in the cornea

To assess the effect of anti-LT- treatment on proinflammatory cytokine and chemokine production in SK, mRNA was prepared from corneal extracts on day 15 p.i. following treatment that was started on day 6 p.i. As shown in Fig. 5A, samples from animals treated with anti-LT- showed a five-fold reduction of IL-1 when compared to isotype treated animals. Additionally, levels of chemokines involved in neutrophil (KC) and monocyte (MCP-1) migration, lymphocyte migration (CXCL-9 and CXCL-10), as well as IFN- were significantly decreased in anti-LT- treated animals. CTLA-4.Ig treatment had a similar effect as anti-LT-, suggesting that the production of proinflammatory cytokines and chemokines in the cornea of diseased animals was dependent on T cell activation. In contrast, anti-LTMT treatment showed no significant expression differences in most of the proinflammatory molecules when compared to the isotype treated group. Only CXCL-9 showed a significant decrease and even though IFN- was decreased, this reduction was not significant, indicating that depletion of LT- -expressing cells is required for inhibition of proinflammatory responses, but that blockade of LT- 3/TNFR-mediated signaling may have a minimal proinflammatory role in the SK system.

To determine if the reduction in chemokine expression in the corneas of anti-LT- treated mice had functional consequences in terms of impacting migration of infiltrating cells, the frequency and cell numbers of neutrophils in the cornea was analyzed. KC is a chemokine involved in neutrophil recruitment, and neutrophils infiltrating the cornea are responsible for the tissue damage associated with SK [4]. Consistent with the finding that anti-LT- treatment reduced expression of KC, neutrophil frequencies and total cell numbers were significantly lower in the anti-LT- group compared to isotype control (Fig. 5B). Again, anti-LTMT showed a pattern of reduction in frequencies and numbers of neutrophils when compared to the isotype treated animals but this reduction was not significant.

4. Discussion

Stromal keratitis is a blinding lesion of the eye set off by HSV-1 with its pathogenesis primarily involving events that are common to several autoimmune diseases. Indeed, some groups have strongly advocated that the syndrome could involve autoreactive events, but the exact mechanisms that occur, particularly in human lesions, have not been resolved [4, 5]. Many chronic tissue lesions are controlled using powerful drugs such as corticosteroids that have unwanted side effects [22, 23]. More acceptable approaches to therapy could come from targeting specific cells that contribute to tissue damage. In the blinding lesion that results from HSV infection, damage to the cornea is thought to result from a T cell orchestrated reaction set off by the infection [3, 5, 24].

Accordingly, a logical approach to therapy could be to target the pathological T cells and inhibit their activity. In this report we show that this objective can be met by administering a mAb specifically recognizing LT- during ongoing lesions. We showed that anti-LT- mAb treatment significantly reduced SK lesions and markedly reduced the numbers of inflammatory cells and their products in the ocular responses, and this effect was similar to blockade of T cell activation with CTLA-4.Ig. The effect was presumed to target primarily LT- inflammatory CD4⁺ T cells, particularly the Th1 subset, since a mAb lacking the Fc receptor binding activity, that cannot mediate targeted depletion, had reduced therapeutic activity even though the mutant mAb could neutralize the proinflammatory effects of the soluble LT- homotrimer *in vitro*. Our results indicate that treatment with mAb to LT- can beneficially influence the outcome of ongoing SK lesions in a model system with the treatment holding promise as a new approach to control an important cause of human blindness.

The biology of LT-3 is intriguing in the context of SK. Soluble LT-3 binds to TNFR1 and TNFR2, and ligation initiates signaling through the canonical NF- κ B pathway which controls many inflammatory genes [25]. Membrane-bound LT-1/2 binds to LT-R, with engagement activating both the canonical and non-canonical NF- κ B pathways [25]. Unlike TNF, which is produced by a wide array of cells, LT-3 expression is restricted and predominantly produced by activated CD4⁺ Th cells, namely Th1 and Th17 cells. Similarly, LT-1/2 is expressed on the surface of these Th subsets, and serves as a marker identifying these populations. Thus, LT-3 can induce production of inflammatory cytokines and chemokines either through direct interaction between cells expressing LT-1/2 with those expressing LT-R within the cornea such as fibroblasts, epithelial cells and myeloid cells. Additionally, soluble LT-3 can exert its influence distally on ubiquitously expressed TNFR1 or the more restricted TNFR2.

We utilized a depleting anti-LT-3 mAb that has the capacity to bind both LT-3 and LT-1/2 as well as block LT-3/TNFR signaling to evaluate whether LT itself has a role in mediating SK or if LT-producing cells are critical. We showed a significant therapeutic effect of anti-LT-3 when used to treat SK and demonstrated that it is the pathogenic T cells, and not their production of LT-3, that are responsible for SK. We were able to demonstrate that LT-3 as well as LT-1/2 can induce the production of proinflammatory chemokines *in vitro* in a cell line that is derived from mouse keratocytes, indicating that direct LT cytokine interactions with their cognate receptors may have a role in SK pathology. While the anti-LTMT was similar to wild-type mAb in its ability to inhibit LT-3-induced activation of corneal stromal fibroblasts *in vitro*, anti-LTMT treatment did not ameliorate SK lesion severity to a significant degree, indicating that blockade of LT-3/TNFR interactions is not sufficient to therapeutically impact disease. Instead, the efficacy of wild-type anti-LT-3 was not as clearly seen with the mutant Ab, suggests that LT-3-expressing cells were specifically targeted for depletion. CD4⁺ T cell numbers, particularly the Th1 subset, were significantly reduced in anti-LT-3 treated animals. Treatment with anti-LTMT showed a tendency towards reduction of Th1 cells in the cornea and DLN when compared to the isotype treated animals, however this did not achieve statistical significance. We were not able to show full depletion or conclude if the effects of anti-LT-3 were directly occurring in the cornea or due to peripheral deletion of Th1 cells resulting in fewer cells available to migrate into the cornea. While we attempted to directly measure depletion of LT-3-expressing cells by flow cytometry of cells harvested from corneas *ex vivo*, this proved to be challenging. Previous reports demonstrated that depletion of LT-expressing cells could be observed in *in vitro* recall assays using specific antigens [17], a technique not available to us with the SK model. Our contention that depletion of pathogenic cells expressing surface LT is responsible for efficacy of anti-LT-3 is bolstered by similar findings in other models of inflammation such as collagen-induced arthritis, delayed-type hypersensitivity and experimental autoimmune encephalomyelitis [17].

While it is generally accepted that Th1 cells are the primary mediators of the inflammatory reaction, neutrophils are primarily responsible for causing the actual tissue damage [4]. Anti-LT-3 treatment significantly reduced corneal expression of various chemokines, including the neutrophil chemoattractant KC. Correspondingly, neutrophil numbers were also found to be lower in anti-LT-3 treated corneas. Even though numbers of neutrophils were decreased in the anti-LT-3 treated group, we believe neutrophils were not depleted by the Ab, given the fact that they do not express LT-3 on their surface data not shown. Contrary, we believe this is likely due to the anti-LT-3 mediated reduction of pathogenic Th1 cells and not direct depletion of neutrophils or inhibition of the production of proinflammatory cytokines that attract neutrophils by LT-3, as CTLA-4.Ig similarly impacted both KC expression and neutrophil infiltration. The ability of anti-LT-3 to eliminate activated Th1 cells and of CTLA-4.Ig to inhibit activation of Th1 cells [20] results

in similar outcomes: a reduction in the secretion of Th1 proinflammatory factors that drive downstream pathogenic signals such as the production of proinflammatory cytokines and chemokines and therefore the infiltration of other proinflammatory cells important in the pathogenesis of SK, an activity which anti-LTMT lacks.

The anti-LT- mAb used in these studies did not allow us to assess the role of LT- 1 2 interaction with LT- R in SK. Unlike its ability to completely block LT- 3/TNFR, this mAb, as well as the Fc mutant, was shown to only partially block LT- 1 2/LT- R [17]. MK/T-1 cells responded to *in vitro* stimulation with LT- 1 2 with robust production of chemokines, suggesting that LT- 1 2-expressing CD4⁺ T cells infiltrating the corneas upon infection with HSV-1 can generate a milieu of proinflammatory cytokines and chemokines through direct contact with stromal cells, leading to infiltration of pathogenic cells that results in lesions associated with SK. As treatment with anti-LT- mAb depletes those cells expressing surface LT- 1 2, the potential for LT- 1 2 binding to LT- R is also eliminated. However, since the ability of TNFR1 to induce proinflammatory responses is relatively stronger than that of LT- R [26], it is unlikely that the LT- 1 2/LT- R pathway significantly contributes to pathology given that LT- 3/TNFR does not.

In addition to Th1 cells, Th17 cells also express LT- 1 2 on the cell surface and secrete LT- 3 [17]. However, we did not focus on Th17 responses since in the SK model, Th17 cells appear to play only a minor role in SK pathogenesis, and these cells never outnumber Th1 [27]. Our observations add further evidence to the notion that Th1 cells play a critical role during HSV-1 infection. We can also infer from our data that therapies that target Th1 responses in the phase of active lesions could be beneficial to the outcome of the disease. Unlike CTLA-4.Ig, which broadly inhibits all T cell activation, anti-LT- mAb only depletes activated pathogenic Th cells. Moreover, anti-LT- also targets the soluble fraction of LT- . LT- 3 may have some proinflammatory activity in the SK system as shown *in vitro* and since blocking it with anti-LTMT treatment *in vivo* somehow reduced the severity of disease even though this reduction was not significant. The ability of anti-LT- to specifically deplete pathogenic Th cells and to eliminate LT- 3 makes it a better candidate to treat SK.

Regulatory T cells (Tregs) also play an important role in protecting the host from ocular immunopathology [29] and expanding their numbers can result in diminished SK. Moreover, Tregs do not express LT- 1 2, and therefore are not depleted by the administration of anti-LT- [17]. Combination therapies using anti-LT- and a drug that could boost or expand Treg responses [12, 30-32] could prove even more efficacious in controlling and diminishing SK lesions, and these studies are currently being evaluated.

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References

- [1]. Pepose, JD. Herpes simplex virus disease: Anterior segment of the eye. Mosby; St. Louis: 1996.
- [2]. Giménez F, Suryawanshi A, Rouse BT. Pathogenesis of herpes stromal keratitis - A focus on corneal neovascularization. Prog Retin Eye Res. 2012;1–9. [PubMed: 22892644]

- [3]. Streilein JW, Dana MR, Ksander BR. Immunity causing blindness: five different paths to herpes stromal keratitis. *Immunol Today*. 1997; 18:443–449. [PubMed: 9293161]
- [4]. Biswas PS, Rouse BT. Early events in HSV keratitis--setting the stage for a blinding disease. *Microbes Infect*. 2005; 7:799–810. [PubMed: 15857807]
- [5]. Zhao ZS, Granucci F, Yeh L, Schaffer PA, Cantor H. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science*. 1998; 279:1344–1347. [PubMed: 9478893]
- [6]. Dawson CR, Togni B. Herpes simplex eye infections: clinical manifestations, pathogenesis and management. *Surv Ophthalmol*. 1976; 21:121–135. [PubMed: 988644]
- [7]. Metcalf JF, Kaufman HE. Herpetic stromal keratitis-evidence for cell-mediated immunopathogenesis. *Am J Ophthalmol*. 1976; 82:827–834. [PubMed: 187060]
- [8]. Russell RG, Nasisse MP, Larsen HS, Rouse BT. Role of T-lymphocytes in the pathogenesis of herpetic stromal keratitis. *Invest Ophthalmol Vis Sci*. 1984; 25:938–944. [PubMed: 6611324]
- [9]. Knotts FB, Cook ML, Stevens JG. Pathogenesis of herpetic encephalitis in mice after ophthalmic inoculation. *J Infect Dis*. 1974; 130:16–27. [PubMed: 4366287]
- [10]. Niemialtowski MG, Rouse BT. Predominance of Th1 cells in ocular tissues during herpetic stromal keratitis. *J Immunol*. 1992; 149:3035–3039. [PubMed: 1357034]
- [11]. Hendricks RL, Tumpey TM, Finnegan A. IFN-gamma and IL-2 are protective in the skin but pathologic in the corneas of HSV-1-infected mice. *J Immunol*. 1992; 149:3023–3028. [PubMed: 1401927]
- [12]. Sehrawat S, Rouse BT. Anti-inflammatory effects of FTY720 against viral-induced immunopathology: role of drug-induced conversion of T cells to become Foxp3+ regulators. *J Immunol*. 2008; 180:7636–7647. [PubMed: 18490766]
- [13]. Rajasagi NK, Suryawanshi A, Sehrawat S, Reddy PB, Mulik S, Hirashima M, Rouse BT. Galectin-1 reduces the severity of herpes simplex virus-induced ocular immunopathological lesions. *J Immunol*. 2012; 188:4631–4643. [PubMed: 22467659]
- [14]. Deshpande SP, Zheng M, Lee S, Rouse BT. Mechanisms of pathogenesis in herpetic immunoinflammatory ocular lesions. *Vet Microbiol*. 2002; 86:17–26. [PubMed: 11888686]
- [15]. Gommerman JL, Browning JL. Lymphotoxin/light, lymphoid microenvironments and autoimmune disease. *Nat Rev Immunol*. 2003; 3:642–655. [PubMed: 12974479]
- [16]. Chiang EY, Kolumam G, McCutcheon KM, Young J, Lin Z, Balazs M, Grogan JL. In vivo depletion of lymphotoxin-alpha expressing lymphocytes inhibits xenogeneic graft-versus-host-disease. *PLoS One*. 2012; 7:e33106. [PubMed: 22427961]
- [17]. Chiang EY, Kolumam GA, Yu X, Francesco M, Ivelja S, Peng I, Gribbling P, Shu J, Lee WP, Refino CJ, Balazs M, Paler-Martinez A, Nguyen A, Young J, Barck KH, Carano RA, Ferrando R, Diehl L, Chatterjea D, Grogan JL. Targeted depletion of lymphotoxin-alpha-expressing TH1 and TH17 cells inhibits autoimmune disease. *Nat Med*. 2009; 15:766–773. [PubMed: 19561618]
- [18]. Suryawanshi A, Veiga-Parga T, Reddy PB, Rajasagi NK, Rouse BT. IL-17A differentially regulates corneal vascular endothelial growth factor (VEGF)-A and soluble VEGF receptor 1 expression and promotes corneal angiogenesis after herpes simplex virus infection. *J Immunol*. 2012; 188:3434–3446. [PubMed: 22379030]
- [19]. Suryawanshi A, Mulik S, Sharma S, Reddy PB, Sehrawat S, Rouse BT. Ocular neovascularization caused by herpes simplex virus type 1 infection results from breakdown of binding between vascular endothelial growth factor A and its soluble receptor. *J Immunol*. 2011; 186:3653–3665. [PubMed: 21325621]
- [20]. Gangappa S, Manickan E, Rouse BT. Control of herpetic stromal keratitis using CTLA4Ig fusion protein. *Clin Immunol Immunopathol*. 1998; 86:88–94. [PubMed: 9434800]
- [21]. Shields RL, Namenuk AK, Hong K, Meng YG, Rae J, Briggs J, Xie D, Lai J, Stadlen A, Li B, Fox JA, Presta LG. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem*. 2001; 276:6591–6604. [PubMed: 11096108]
- [22]. Deshpande S, Banerjee K, Biswas PS, Rouse BT. Herpetic eye disease: immunopathogenesis and therapeutic measures. *Expert Rev Mol Med*. 2004; 6:1–14. [PubMed: 15061872]

- [23]. Knickelbein JE, Hendricks RL, Charukamnoetkanok P. Management of herpes simplex virus stromal keratitis: an evidence-based review. *Surv Ophthalmol*. 2009; 54:226–234. [PubMed: 19298901]
- [24]. Biswas PS, Banerjee K, Kim B, Kinchington PR, Rouse BT. Role of inflammatory cytokine-induced cyclooxygenase 2 in the ocular immunopathologic disease herpetic stromal keratitis. *J Virol*. 2005; 79:10589–10600. [PubMed: 16051851]
- [25]. Ware CF. Network communications: lymphotoxins, LIGHT, and TNF. *Annu Rev Immunol*. 2005; 23:787–819. [PubMed: 15771586]
- [26]. Hochman PS, Majeau GR, Mackay F, Browning JL. Proinflammatory responses are efficiently induced by homotrimeric but not heterotrimeric lymphotoxin ligands. *J Inflamm*. 1995; 46:220–234. [PubMed: 8878796]
- [27]. Suryawanshi A, Veiga-Parga T, Rajasagi NK, Reddy PB, Sehrawat S, Sharma S, Rouse BT. Role of IL-17 and Th17 cells in herpes simplex virus-induced corneal immunopathology. *J Immunol*. 2011; 187:1919–1930. [PubMed: 21765013]
- [28]. Young J, Yu X, Wolslegel K, Nguyen A, Kung C, Chiang E, Kolumam G, Wei N, Wong WL, DeForge L, Townsend MJ, Grogan JL. Lymphotoxin- α heterotrimers are cleaved by metalloproteinases and contribute to synovitis in rheumatoid arthritis. *Cytokine*. 2010; 51:78–86. [PubMed: 20356761]
- [29]. Veiga-Parga T, Suryawanshi A, Mulik S, Giménez F, Sharma S, Sparwasser T, Rouse BT. On the role of regulatory T cells during viral-induced inflammatory lesions. *J Immunol*. 2012; 189:5924–5933. [PubMed: 23129753]
- [30]. Reddy PB, Schreiber TH, Rajasagi NK, Suryawanshi A, Mulik S, Veiga-Parga T, Niki T, Hirashima M, Podack ER, Rouse BT. TNFRSF25 agonistic antibody and galectin-9 combination therapy controls herpes simplex virus-induced immunoinflammatory lesions. *J Virol*. 2012; 86:10606–10620. [PubMed: 22811539]
- [31]. Sehrawat S, Suvas S, Sarangi PP, Suryawanshi A, Rouse BT. In vitro-generated antigen-specific CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells control the severity of herpes simplex virus-induced ocular immunoinflammatory lesions. *J Virol*. 2008; 82:6838–6851. [PubMed: 18480441]
- [32]. Veiga-Parga T, Suryawanshi A, Rouse BT. Controlling viral immuno-inflammatory lesions by modulating aryl hydrocarbon receptor signaling. *PLoS Pathog*. 2011; 7:e1002427. [PubMed: 22174686]

FIGURE 1. Changes in LT- α and LT- β as a consequence of HSV-1 infection

WT mice corneas were scarified and infected with 10^4 PFU HSV-1 in PBS or mock infected with only PBS (scratched uninfected control mice). Corneas were harvested from infected mice at indicated time points p.i. and from mock infected mice at 24 hours p.i. (A) Kinetic analysis for the expression of LT- α mRNA by Q-RT-PCR at different days p.i. At each time point, six corneas were collected and pooled for mRNA extraction. The expression levels of LT- α were normalized to β -actin using C_t calculation. Relative expression between control and infected groups was calculated using the 2^{-C_t} formula. Data are representative of three independent experiments and show mean values $\pm$ SEM (each sample is representative of 6 corneas). (B) Kinetic analysis for the expression of LT- β mRNA by Q-RT-PCR at different days p.i. At each time point, six corneas were collected and pooled for mRNA extraction. The expression levels of LT- β were normalized to β -actin using C_t calculation. Relative expression between control and infected groups was calculated using the 2^{-C_t} formula. Data are representative of three independent experiments and show mean values $\pm$ SEM (each sample is representative of 6 corneas).

FIGURE 2. LT- 3 and LT- 1 2 induce the expression of proinflammatory cytokines by corneal stromal fibroblast cells

(A) MK/T-1 cells were stimulated with 100ng/ml of LT- 3 or media alone for 6 h. MK/T-1 were also stimulated with 100ng/ml of LT- 3 in the presence of anti-LT- or anti-LTMT at 0.1 μ M. Cells were collected and pooled for analysis of KC and CXCL-10 mRNA expression by Q-RT-PCR. Statistical levels of significance were analyzed by one-way ANOVA test with Dunnett's post hoc test settings. (B) MK/T-1 cells were stimulated with 100ng/ml of LT- 1 2 or media alone for 6 h. MK/T-1 were also stimulated with 100ng/ml of LT- 1 2 in the presence of anti-LT- or anti-LTMT at 0.1 μ M. Cells were collected and pooled for analysis of KC and CXCL-10 mRNA expression by Q-RT-PCR. Statistical levels of significance were analyzed by an unpaired two-tailed Student t test.. Data are representative of three independent experiments and show mean values $\pm$ SEM. *** p < 0.001, ** p < 0.01, * p < 0.05.

Mice infected with HSV-1 were given either isotype control, LT- α -specific mAb, anti-LTMT or CTLA-4.Ig s.c. starting on day 6 p.i., and treated 3 times a week until the termination day (day 15 p.i.). **(A)** SK lesion severity was significantly decreased in the groups of mice treated with anti-LT- α when compared to isotype or anti-LTMT treated mice on day 15 p.i. Individual eye scores of SK lesion severity on day 15 p.i. are shown. **(B)** Representative eye photos show decreased SK lesion severity from anti-LT- α treated mice starting on day 6 p.i. compared with isotype and anti-LTMT treated mice. **(C)** Eyes were processed for cryo-sections on day 15 p.i. Hematoxylin and eosin staining was carried out

on 6- μ m sections, and pictures were taken 40 $\times$ magnification. Representative eye sections show decreased cellular infiltration in anti-LT- treated mice when compared to isotype and anti-LTMT treated animals. Data are representative of three independent experiments and show mean values $\pm$ SEM (n = 12-15 mice/group). *** p 0.001, ** p 0.01, * p 0.05. Statistical levels of significance were analyzed by one-way ANOVA test with Dunnett's post hoc test settings.

FIGURE 4. LT- α -specific mAb treatment decreases cellular infiltration in corneas of HSV-1 infected animals

Mice infected with HSV-1 were treated with isotype control, anti-LT- α , anti-LTMT or CTLA-4.Ig starting on day 6 p.i., 3 days a week until the termination day (day 15 p.i.). (A) Representative FACS plots and numbers of CD4⁺ T cells isolated from corneas of treated mice are shown. (B). Representative FACS plots and numbers of IFN- γ -secreting cells gated on CD4⁺ T cells infiltrated in the cornea on day 15 p.i. and stimulated with anti-CD3/anti-CD28 for 5 h. (C) Representative FACS plots and numbers of IFN- γ -secreting cells gated on CD4⁺ T cells infiltrated in DLN on day 15 p.i. and stimulated with anti-CD3/anti-CD28 for 5 h. Data are representative of three independent experiments and show mean values $\pm$ SEM ($n = 10$ -12 mice/group). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Statistical levels of significance were analyzed by one-way ANOVA test with Dunnett's post hoc test settings.

FIGURE 5. LT- α -specific mAb treatment decreases cytokine and chemokine levels and neutrophil infiltration in corneas of HSV-1-infected animals

Mice infected with HSV-1 were given either isotype, anti-LT- α , anti-LTMT, or CTLA-4.Ig starting on day 6 p.i. 3 times a week until the termination day (day 15 p.i.). **(A)** Mice were sacrificed on day 15 p.i., and corneal extracts were collected for measuring inflammatory factors using Q-RT-PCR. Relative fold change in mRNA expression of the proinflammatory cytokine IL-1 β , of the chemokines KC, MCP-1, CXCL9, and CXCL10, and of the proinflammatory molecule IFN- γ were examined. mRNA levels for the different cytokines in mock-infected mice were set to one and used for the relative fold upregulation. Data are representative of three independent experiments and show mean values $\pm$ SEM ($n = 12$ mice/group, each sample is representative of 4 corneas). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Statistical levels of significance were analyzed by one-way ANOVA test with Dunnett's post hoc test settings. **(B)** Representative FACS plots and numbers of CD11b⁺Ly6G⁺ polymorphonuclear neutrophils gated on total CD45⁺ cells isolated from corneas of treated mice are shown. Data are representative of three independent experiments and show mean values $\pm$ SEM ($n = 10$ -12 mice/group). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Statistical levels of significance were analyzed by one-way ANOVA test with Dunnett's post hoc test settings.