Modulation of Integrin $\alpha_4\beta_1$ (VLA-4) in Dry Eye Disease

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Objective: To study the effect of topical application of very late antigen 4 (VLA-4) small-molecule antagonist (anti–VLA-4 sm) in a mouse model of dry eye disease.

Methods: Anti–VLA-4 sm (or control vehicle) was applied topically to mice placed in a controlled-environment chamber. Corneal fluorescein staining and conjunctival T-cell enumeration were performed in the different treatment groups. Real-time polymerase chain reaction was used to quantify expression of inflammatory cytokines in the cornea and conjunctiva.

Results: Dry eye syndrome induced increased corneal fluorescein staining, corneal and conjunctival tumor necrosis factor $\alpha$ messenger RNA expression, and T-cell infiltration into the conjunctiva. Very late antigen 4 blockade significantly decreased corneal fluorescein staining compared with the untreated dry eye disease and control vehicle–treated groups ($P < .001$ and $P = .02$, respectively). In addition, VLA-4 blockade was associated with a significant decrease in conjunctival T-cell numbers ($P < .001$ vs control vehicle–treated group) and tumor necrosis factor-$\alpha$ transcript levels in the cornea ($P = .04$ vs control vehicle–treated group) and conjunctiva ($P = .048$ vs control vehicle–treated group).

Conclusion: Application of topical anti–VLA-4 sm led to a significant decrease in dry eye signs and suppression of inflammatory changes at the cellular and molecular levels.

Clinical Relevance: Topical blockade of VLA-4 may be a novel therapeutic approach to treat the clinical signs and inflammatory changes accompanying dry eye disease.

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Dry eye disease (DED) or keratoconjunctivitis sicca is a common ocular surface disease affecting tens of millions of people worldwide. It is one of the leading reasons for patients seeking eye care for treatment of symptoms of ocular discomfort and visual disturbance and commonly leads to problems with activities that require sustained visual attention such as reading and driving. To date, the pathogenesis of DED is not fully understood, but recent studies have demonstrated that DED is associated with ocular surface inflammation, which contributes to the disease process and the symptoms. Therefore, targeting molecules involved in mediating inflammation could be a promising approach for the treatment of DED.

Inflammation is a complex response to local injury or trauma involving various immunocytes and numerous mediators. The assembly of the inflammatory response would be impossible without the controlled migration of leukocytes, of which cell adhesion molecules such as integrins are key components for migration and activation of these cells. Integrins are heterodimeric molecules and cell surface transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions. Very late antigen 4 (VLA-4 [integrin $\alpha_4\beta_1$]) exerts its functions through interaction with its ligands fibronectin, vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cell adhesion molecule 1. It has been demonstrated that VLA-4 can serve as a target for treatment of inflammatory disorders. For example, blocking of VLA-4 with monoclonal antibody has been shown to reduce relapses of multiple sclerosis. The use of anti-$\alpha_4$ monoclonal antibody has also been demonstrated to be effective in suppressing endotoxin-induced uveitis and inhibiting eosinophil infiltration in a guinea pig model of allergic conjunctivitis. Recently, VLA-4 blockade with a peptide inhibitor has also led to inhibition of the development of experimental autoimmune uveitis. Although inflammation has been shown to be related to the pathogenesis of dry eye disease, topical blockade of VLA-4 or other integrins has not been investigated in DED, to our knowledge. Therefore, we examined the therapeutic effects of VLA-4 small-molecule antagonist (anti–VLA-4 sm) on dry eye using the controlled-environment chamber (CEC) model, which reliably induces signs of DED in mice.

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MOUSE MODEL OF DED

The protocol was approved by the institutional animal care and use committee, and all animals were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Sixto-eight-week-old female C57Bl/6 mice (Taconic Farms, Germantown, New York) were used in these experiments. Dry eye disease was induced by exposure of mice to a CEC11 for 10 days during which mice also received administration of scopolamine hydrobromide to maximize ocular dryness. Briefly, scopolamine hydrobromide (0.5 mg/0.2 mL; Sigma-Aldrich Inc, St Louis, Missouri) was injected subcutaneously in the dorsal skin of mice 4 times daily. The mice placed in the CEC were continuously exposed to a relative humidity below 30%, an airflow of 15 L/min, and a constant temperature (21°C-23°C) as previously described.11 Age-matched mice that were kept in a normal non-desiccating environment (relative humidity >70%, no additional airflow, temperature 21°C-23°C, and no scopolamine administration) were used as normal controls.

ANTI–VLA-4 SM FORMULATION AND TREATMENT REGIMEN

BIO-8809 (anti–VLA-4 sm) and a control compound (BIO-9257) were commercially obtained (Biogen Idec, Inc, Cambridge, Massachusetts). BIO-8809 is the pegylated, more stable version of (2S)-(1-(3,5-dichloro-benzenesulfonyl)-pyrrolidine-2(S)-carbonyl-amino)-4-[4-methyl-2(S)-(methyl-[2-[4-(3-octyl-ureido)-phenyl]-acetyl]-amino)-pentanoylamino]-butyric acid, which has been identified as a highly selective and potent (Kd=9 pM [picomolars]) small-molecule antagonist to integrin αβ4 (VLA-4).13 BIO-8809 was in a Tris-lactose buffer (15 mg/mL [pH 7.5]). To control for the exposure of the eyes to this pegylated compound, BIO-9257 was used as a control consisting of the pegylated conjugate of (2S,4R)-4-[6-(3-hydroxypropanamido)-hexanamido]-1-[(phenylsulfonyl) pyrrolidine-2-carboxylic acid, which is an inactive compound with physicochemical properties similar to those of BIO-8809 in a Tris-lactose buffer (pH 7.5).

Forty-eight hours after dry eye induction, mice were randomized to receive anti–VLA-4 sm BIO-8809 or control vehicle BIO-9257 on both eyes. An eyedrop (5 µL) was topically applied to the eyes of the unanesthetized mice twice a day from day 2 to day 10. The untreated dry eye group received no eyedrops. Ocular signs of dry eye disease were measured at day 2, day 5, day 7, and day 10. Mice were then euthanized at day 10 for cellular and molecular studies.

MEASUREMENT OF CORNEAL FLUORESCINE STAINING

Corneal fluorescent staining was performed at baseline (day 0) and then at day 2 (before administration of the first eyedrop dose), day 5, day 7, and day 10. Fluorescein (5% [1 µL]) was applied to the lateral conjunctival sac of the mice as previously described.13 Eyes were examined for fluorescent staining after 5 minutes using a slitlamp biomicroscope under cobalt blue light. Punctate staining was recorded in a masked fashion using the standard National Eye Institute grading system,14 giving a score from 0 to 3 for each of 5 areas of the cornea.

IMMUNOHISTOCHEMICAL STAINING

The following antibodies were used for immunohistochemical staining: purified hamster antimouse CD3e monoclonal an-{}


tobody (T-cell marker, catalog No. 553057; and isotype control purified hamster IgG1, catalog No. 11121D), secondary antibody Cy-3 goat anti-Armenian hamster antibody (catalog No. 127135-160), and fluorescein isothiocyanate–conjugated (FITC) rat antimouse CD4 (catalog No. 553046; and isotype FITC rat IgG2a, catalog No. 553929). All primary antibodies and isotype-matched controls were purchased from BD Pharmingen, San Diego, California; secondary antibody was purchased from Jackson Laboratories, Bar Harbor, Maine.

For cross-sectional staining of the conjunctiva, whole eyeballs were excised. They were frozen in OCT, cut into 7-µm frozen sections, and fixed in acetone for 15 minutes at room temperature. All antibodies were diluted in 2% bovine serum albumin. To block nonspecific staining, cross sections were blocked with anti-Fc monoclonal antibody CD16/CD32 (catalog No. 553142, BD Pharmingen) for 30 minutes. Next, the tissues were immunostained with primary antibodies or isotype-matched control antibodies overnight at 4°C. After 3 thorough washings with phosphate-buffered saline (PBS) for 10 minutes each, cross sections were incubated with the secondary antibody for 1 hour at room temperature. The cross sections were then washed in PBS again 3 times and were mounted using mounting medium (Vector Shield, Vector Laboratories, Burlingame, California). Three eyeballs from 3 mice per group were used for T-cell enumeration in the bulbar and tarsal conjunctivae. Serial cross sections per slide were studied under an epifluorescence microscope (model E800; Nikon, Melville, New York) with a ×40 objective lens. The mean number of cells was obtained by averaging the cell numbers per cross sections studied.

RNA ISOLATION, REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION, AND REAL-TIME POLYMERASE CHAIN REACTION

Total RNA from whole-mount corneas was isolated using a microkit (RNeasy; Qiagen, Studio City, California) and was stored at −80°C until future use. The first strand of complementary DNA (cDNA) was synthesized with random hexamers using a transcriptase (SuperScriptIII Reverse Transcriptase; Invitrogen, San Diego, California) according to the manufacturer’s protocol. Real-time polymerase chain reaction (PCR) was performed with FAM-MGB dye-labeled predesigned primers (Applied Biosystems, Foster City, California) for tumor necrosis factor (TNF)-α (assay ID. Mm99999908_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID. Mm99999915_g1), CD40 (assay ID. Mm00441891_m1), interferon γ (IFN-γ)–inducible protein 10 (or CXCL10 [IP-10]) (assay ID. Mm00445235_m10), and VCAM-1 (assay ID. Mm00449197_m10) according to the manufacturer’s recommendations. One microliter of cDNA was loaded in each well, and assays were performed in duplicates. A nontemplate control was included in all experiments to evaluate DNA contamination. The results of quantitative real-time PCR were analyzed using the comparative threshold cycle method and were normalized to GAPDH as an internal control.

STATISTICAL ANALYSIS

We compared the changes in corneal fluorescent staining scores between treatment groups over time using repeated-measures analysis of variance. For the outcomes of total conjunctival T cells, TNF messenger RNA (mRNA) levels, and transcript levels of IP-10, CD40, and VCAM-1, comparisons between pairs of treatment groups were performed using Mann-Whitney test (InStat3 software; Graphpad Software, Inc, La Jolla, California).
Minimal or no corneal punctate staining was observed at day 0. At day 2, all mice showed increased corneal fluorescein staining, corresponding to dry eye induction in the CEC model. Topical treatment was started at day 2, followed by evaluation of the differences in corneal fluorescein staining scores between the groups at day 5 vs day 2, day 7 vs day 2, and day 10 vs day 2 (Figure 1). The control compound (BIO-9257)–treated group (n=16) and the anti–VLA-4 sm–treated group (n=16) had staining scores that decreased with time compared with the untreated dry eye group (n=11). However, the anti–VLA-4 sm–treated group significantly differed in the magnitude of the staining decrease compared with the untreated dry eye group (P<.001 for trend) and the control BIO-9257–treated group (P=.02 for trend).

**RESULTS**

**CORNEAL FLUORESCINE STAINING IN DRY EYES TREATED WITH TOPICAL BLOCKADE OF VLA-4**

Possible changes based on therapy were assessed (Figure 2). The total number of CD3+ T cells was significantly increased in the untreated dry eye group compared with the normal group (P<.001). This trend was observed for CD4+ (P=.01) and CD4− (P=.004) T cells. Application of topical anti–VLA-4 sm led to a significant decrease in the total number of conjunctival T cells compared with that in the untreated dry eye group and the control BIO-9257–treated group (14-21% decrease per group [P<.001 for both]). The effect of VLA-4 blockade on T-cell infiltration was evident for CD4+ T cells (P<.001 vs untreated DED group and vs BIO-9257–treated group) and for CD4− T cells (P<.001 vs untreated DED group; P=.01 vs BIO-9257–treated group). In contrast, the effect of topical application of BIO-9257 on T-cell infiltration was evident for CD4+ T cells (P=.02 vs untreated DED group) but not for CD4− T cells (P=.49 vs untreated DED group) or total CD3+ T cells (P=.15 vs untreated DED group).

**ENUMERATION OF CD3+ T CELLS IN THE CONJUNCTIVA**

 Conjunctival infiltration by T cells is a hallmark of clinically significant DED.3,4 Accordingly, CD3+ T cells were enumerated in the conjunctiva of dry eye mice, and the changes based on therapy were assessed (Figure 2). The total number of CD3+ T cells was significantly increased in the untreated dry eye group compared with the normal group (P<.001). This trend was observed for CD4+ (P=.01) and CD4− (P=.004) T cells. Application of topical anti–VLA-4 sm led to a significant decrease in the total number of conjunctival T cells compared with that in the untreated dry eye group and the control BIO-9257–treated group (14-21% decrease per group [P<.001 for both]). The effect of VLA-4 blockade on T-cell infiltration was evident for CD4+ T cells (P<.001 vs untreated DED group and vs BIO-9257–treated group) and for CD4− T cells (P<.001 vs untreated DED group; P=.01 vs BIO-9257–treated group). In contrast, the effect of topical application of BIO-9257 on T-cell infiltration was evident for CD4+ T cells (P=.02 vs untreated DED group) but not for CD4− T cells (P=.49 vs untreated DED group) or total CD3+ T cells (P=.15 vs untreated DED group).

**EXPRESSION OF TNF-α IN THE CORNEA AND CONJUNCTIVA**

Real-time PCR was used as a quantitative technique to evaluate the levels of RNA transcripts encoding TNF-α and IFN-γ in the cornea. No significant changes among the groups were observed in IFN-γ mRNA levels (data not shown). However, induction of DED led to a 1.7-fold increase in TNF-α expression in the cornea by day 10 (Figure 3). Application of topical anti–VLA-4 sm (n=10) significantly decreased TNF-α expression compared with that in the untreated DED group (n=9) (P=.008) and the BIO-9257–treated group (n=10) (P=.04), with a final value for TNF-α mRNA at day 10 in the anti–VLA-4 sm–treated group close to that seen in normal control eyes. The same trend was observed in the conjunctiva, with a significant decrease in TNF-α transcripts in the anti–VLA-4 sm–treated group (n=10) vs the BIO-9257–treated group (n=19) (P=.048).

**EXPRESSION OF CONJUNCTIVAL IP-10, CD40, AND VCAM-1 mRNA**

Real-time PCR was performed to quantify the level of RNA transcripts encoding IP-10, CD40, and VCAM-1 in the conjunctiva of mice in the different groups (Figure 4). The mRNA levels of IP-10 were increased 8.2 fold; CD40, 2.4 fold; and VCAM-1, 1.6 fold in the untreated DED group (n=5) compared with those in normal mice (n=5). The expression levels of these 3 markers decreased in the BIO-9257–treated (n=7) and BIO-8809–treated (n=11) groups. However, the most noticeable suppression in the transcript levels was seen in the anti–VLA-4 sm–treated group.

**COMMENT**

The past decade of research has shed light on the importance of inflammation as a component of DED in rodent models3,4 and in humans.5 Lymphocytic infiltration of the lacrimal gland by T cells has been described in Sjögren syndrome13 and in non-Sjögren DED.6,17 Most important, studies18,19 have shown that ocular surface inflammation, including conjunctival infiltration by T cells and overexpression of mediators of inflammation, is also present in most patients with keratoconjunctivitis sicca. This suggests that inflammation, as a cause or as a consequence of DED, is an important facet of the disease process. Given the relevance of VLA-4 in T-cell recruitment and activation, we tested the effect of topical VLA-4 blockade in a murine model of DED. The data presented herein, derived from a masked controlled trial of anti–VLA-4 sm, suggest a significant beneficial effect of VLA-4 blockade in treating the ocular signs and revers-
ing the inflammatory changes of DED at the cellular and molecular levels.

Anti–VLA-4 sm (BIO-8809)–treated eyes demonstrated significant reversal in corneal epithelial damage as manifested by decreased fluorescein staining scores compared with those in the untreated DED and control vehicle–treated groups. The improvement in corneal fluorescein staining, T-cell number, and TNF-α levels seen with the control agent (BIO-9257) is likely due to the lubricating effect of this treatment in the mice with DED. Still, this amelioration is significantly enhanced in the anti–VLA-4 sm–treated group, suggesting that the efficacy observed could not be simply because of the lubricating effect of a topical application. Indeed, VLA-4 has been reported to be expressed in several types of immune and inflammatory cells, including T cells, and the interaction between VLA-4 and its ligand VCAM-1 is important for the migration and infiltration of immune cells.

Figure 2. Enumeration of CD3+ T cells in the conjunctiva. A, Blockade of very late antigen 4 small molecule (VLA-4 sm) significantly decreased the numbers of total conjunctival T cells (\( ^* P < .001 \) vs untreated dry eye disease group; \( ^{\dagger} P < .001 \) vs control BIO-925–treated group), CD4+ T cells, and CD4− T cells (\( ^{\ddagger} P < .01 \) vs control BIO-925–treated group). BIO-9257 also had an effect on CD4+ T cells (\( ^{\ddagger} P < .02 \) vs untreated dry eye disease group). Data represent mean (SEM). B, Representative CD3 (red) and nuclear (blue) immunostaining of conjunctival cross section showing decreased T-cell infiltration (arrows) in the anti–VLA-4 sm–treated group vs the BIO-9257–treated group. Ep indicates epithelial layer; St, stromal layer.

Figure 3. Real-time polymerase chain reaction results showing that very late antigen 4 small-molecule (VLA-4 sm) blockade significantly decreased tumor necrosis factor α (TNF-α) messenger RNA (mRNA) levels compared with those in the BIO-9257–treated control group in the cornea (\( ^{*} P = .04 \)) and in the conjunctiva (\( ^{\ddagger} P = .048 \)). Data represent mean (SEM).

Figure 4. Real-time polymerase chain reaction showing transcript levels of interferon γ (IFN-γ)–inducible protein 10 (IP-10), CD40, and vascular cell adhesion molecule 1 (VCAM-1) in the conjunctivae of the different groups. Data represent mean (SEM). mRNA indicates messenger RNA; VLA-4 sm, very late antigen 4 small molecule.
into inflammatory sites.21 This is in accord with our data suggesting that the therapeutic effect of VLA-4 topical blockade is due, at least in part, to the inhibition of T-cell infiltration into the ocular surface. Indeed, double staining for CD3 and CD4 showed a 3-fold increase in CD4+ T cells but only a 50% increase in CD4− T cells, suggesting a role for the CD4+ subset of T cells in the pathogenesis of DED, consistent with previous findings in humans and in animal models of DED.22-24 VLA-4 blockade was able to suppress T-cell infiltration to levels approaching those seen in normal eyes. Results have demonstrated the immunotherapeutic potential of VLA-4 antagonist in other inflammatory diseases, which has been attributed to the relevance of VLA-4–VCAM-1 interaction in the homing of activated T cells in inflamed tissues,23 consistent with the results of our present study.

Proinflammatory cytokines (such as TNF-α) secreted by T cells or macrophages at sites of inflammation can activate VCAM-1, facilitating adhesion and transendothelial migration of immunocytes from the intravascular compartment to tissue matrices. The precise delineation of the regulation and function of the cytokines, chemokines, and adhesion factors overexpressed in the dry eye state requires further investigation. Notwithstanding this, it is still intriguing that IP-10 is profoundly overexpressed in DED. IP-10 is a CXC chemokine that has a critical role in the recruitment of helper T-cell types 1 and 17.26 It has been demonstrated recently that the interleukin 17F signaling pathway is involved in induction of IP-10 expression in bronchial epithelial cells,27 and the longer and shorter forms of the cytokine can be produced in vivo and in vitro in DED. In addition, IP-10 gene expression in cultured keratoconjunctivitis sicca epithelium is up-regulated by TNF-α and IL-1β.28-30 The suppression of IP-10 gene expression by topical VLA-4 blockade may be a critical mechanism by which VLA-4 antagonist suppresses T-cell infiltration in DED.

Taken together, our data strengthen the concept of an immune-based pathogenesis in DES. In addition, we show for the first time that topical blockade of VLA-4 leads to a significant decrease in the clinical signs of DED and inflammatory changes at the cellular and molecular levels. It is anticipated that ongoing investigations of novel biologic strategies that specifically target pathogenic molecules and pathways involved in DES will lead to more effective therapies for this highly prevalent ocular condition.

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