Modulation of Integrin α₄β₁ (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 α 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-α 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.¹ 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.² To date, the pathogenesis of DED is not fully understood, but recent studies³-⁵ have demonstrated that DED is associated with ocular surface inflammation, which contributes to the disease process and the symptoms.⁶ 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 α₄β₁]) 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 dry eye disease, topical blockade of VLA-4 shown to be related to the pathogenesis of DED, to our knowledge. Therefor, 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.¹¹
butyric acid, which has been identified as a highly selective and
to the lateral conjunctival sac of the mice as previously de-
mine hydrobromide (0.5 mg/0.2 mL; Sigma-Aldrich Inc, St Louis,
hydrobromide to maximize ocular dryness. Briefly, scopolama-
during which mice also received administration of scopolamine
COB-8809 (anti-VLA-4 sm) and a control compound (COB-
Fluorescein staining was performed at baseline (day 0)
and a constant temperature (21°C-23°C) as previously de-
Mm99999915_g1), CD40 (assay ID. Mm00441891_m10), in-
factor (TNF)–inducible protein 10 (or CXCL10 [IP-10])
and assays were performed in duplicates. A nontemplate con-
from Jackson Laboratories, Bar Harbor, Maine.

The protocol was approved by the institutional animal care and
use committee, and all animals were treated according to the As-
s for the Use of Animals in Ophthalmic and Vision Research. Six-
to eight-week-old female C57Bl/6 mice (Taconic Farms, Ger-
machment, New York) were used in these experiments. Dry eye
consumption of the pegylated conjugate of (2-
9257) were commercially obtained (Biogen Idec, Inc, Cam-
consisting of the pegylated conjugate of (2-
and then at day 2 (before administration of the first eyedrop dose),
was injected subcutaneously in the dorsal skin of mice
4 times daily. The mice placed in the CEC were continuously
in a Tris-lactose buffer (pH 7.5).

Forty-eight hours after dry eye induction, mice were ran-
domized to receive anti–VLA-4 sm COB-8809 or control ve-
icle COB-9257 on both eyes. An eyedrop (5 µL) was topical-
ly applied to the eyes of the unanesthetized mice twice a day from
day 2 to day 10. The untreated dry eye group received no eye-
drops. 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 fluorescein 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 de-
scribed.13 Eyes were examined for fluorescein staining after 3 min-
utes using a sliletamp biomicroscope under cobalt blue light. Punct-
tate 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 immunohistochemi-

tibody (T-cell marker, catalog No. 553057; and isotype con-
trol 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 antirat mouse IgG1 (catalog No. 553046; and isotype
FITC rat IgG2ac, catalog No. 553929). All primary antibodies
and isotype-matched controls were purchased from BD Pharmi-
gen, San Diego, California; secondary antibody was pur-
chased from Jackson Laboratories, Bar Harbor, Maine.

Methods

MOUSE MODEL OF DEE

The scheme described.13 Age-matched mice that were kept in a normal non-
desiccating environment (relative humidity >70%, no addi-
tional 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, Cam-
bridge, Massachusetts). BIO-8809 is the pegylated, more stable
version of (S)-[(1-(3,5-dichloro-benzenesulfonyl)-pyrrolidine-
2(S)-carbonyl)-amino]-4-[4-methyl-2(S)-(methyl-[2-[4-(3-o-
tolyl-ureido)-phenyl]-acetyl]-amino]-pentanoylamino]-butyric acid, which has been identified as a highly selective and
potent (Kᵦᵣ = 9 pM [picomolars]) small-molecule antagonist to integrin αβ₄ (VLA-4).12 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-
hydroxypropamido)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).

Total RNA from whole-mount corneas was isolated using a mi-
crokit (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; Invitro-
gen, San Diego, California) according to the manufacturer’s pro-
tocol. Real-time polymerase chain reaction (PCR) was per-
formed with FAM-MGB dye-labeled predesigned primers
(Applied Biosystems, Foster City, California) for tumor necrosis
factor (TNF)–α (assay ID. Mm99999068_m1), glyceraldehyde-
3-phosphate dehydrogenase (GAPDH) (assay ID. Mm00445235_m1),
and assays were performed in duplicates. A nontemplate con-
trol was included in all experiments to evaluate DNA contami-
nation. The results of quantitative real-time PCR were ana-
lyzed using the comparative threshold cycle method and were
normalized to GAPDH as an internal control.

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

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STATISTICAL ANALYSIS

We compared the changes in corneal fluorescein staining scores between treatment groups over time using repeated-
measures analysis of variance. For the outcomes of total con-
njunctival 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).
CORNEAL FLUORESCEIN STAINING IN DRY EYES TREATED WITH TOPICAL BLOCKADE OF VLA-4

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).

ENUMERATION OF CD3+ T CELLS IN THE CONJUNCTIVA

 Conjunctival infiltration by T cells is a hallmark of clinically significant DED.34 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 cross sections 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 group (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 models13,14 and in humans.5 Lymphocytic infiltration of the lacrimal gland by T cells has been described in Sjögren syndrome15 and in non-Sjögren DED.16,17 Most important, studies16,19 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.
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 epithelium,27 and the longer and shorter forms of the IL-17F gene are expressed in activated CD4+ T cells.28,29 The suppression of IP-10 gene expression by topical VLA-4 blockade may be a critical mechanism by which VLA-4 antagonism 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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