Neutralization of Ocular Surface TNF-α Reduces Ocular Surface and Lacrimal Gland Inflammation Induced by In Vivo Dry Eye

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Purpose. The purpose of this study was to investigate the effectiveness of tumor necrosis factor (TNF)-α blocker for treatment of dry eye (DE)-induced inflammation and determine a more effective method to suppress lacrimal gland inflammation. Efficacy of topical versus systemic administration of TNF-α blockers was determined using a murine dry eye (DE) model.

Methods. The TNF-α blocker HL036 was developed by modification of the TNF receptor I. Protein purity, binding affinity, and clearance of TNF-α was compared with etanercept. Using DE-induced C57BL/6 mice, corneal erosion and goblet cell counts were measured after subcutaneous or topical treatment with etanercept or HL036. Inflammatory cytokines in cornea and lacrimal glands were determined by quantitative RT-PCR and ELISA.

Results. HL036 showed TNF-α binding affinity comparable to etanercept, as measured by surface plasmon resonance. HL036 concentration was significantly higher in cornea and anterior segment than etanercept and effectively eliminated TNF-α on ocular surfaces. Etanercept was preferentially concentrated in the posterior segment. Corneal erosion and goblet cell counts were improved only with topically applied etanercept and HL036. Ocular surface IFN-γ, IL-6, and IL-21 were significantly decreased by topical HL036. DE-induced lacrimal gland IFN-γ and IL-6 expression was effectively suppressed by topical etanercept and HL036.

Conclusions. Topical TNF-α blockers effectively suppressed lacrimal gland and corneal inflammation by suppressing IFN-γ, IL-21, and IL-6. Differences in TNF-α affinity, clearance, and local concentration of blockers may account for the anti-inflammatory effects in different ocular regions.

Keywords: dry eye, tumor necrosis factor-α, cytokines, lacrimal gland

Dry eye (DE) syndrome or keratoconjunctivitis sicca (KCS) is one of the most common problems associated with patients at ophthalmology clinics.[1] However, in spite of previous research, the pathogenesis of DE remains unclear. Evidence suggests that cytokines play a central role in DE as the expression of inflammatory cytokines is elevated in affected tissues.[2–4] Studies on various mouse models have reported that T helper cell 1 (Th1), Th2, and Th17 cytokines are critical for the development of dry eye.[5–8] Human studies have demonstrated alterations in protein expression profiles of cytokines on the ocular surfaces of patients with KCS. These include increased concentrations of IL-1α, IL-6, IL-8, IL-12, and TNF-α.[9,10]

TNF-α is a pleiotropic cytokine that has multiple proinflammatory and costimulatory effects on a broad range of cell types.[11] TNF-α regulates cell trafficking, activation, and host defenses against various pathogens, thus playing a major role in orchestrating inflammation and immunity.[11,12] TNF-α antagonists, including the anti-TNF monoclonal antibodies infliximab and adalimumab, the soluble TNF receptor (sTNFRII-Fc), and the drug, etanercept have demonstrated clinical efficacy in treating human rheumatoid arthritis,[11,12] ankylosing spondylitis,[13] psoriatic arthritis,[14] Crohn's disease,[15] and Sjogren's syndrome.[16–18] Sjogren's syndrome is a specific immune type of KCS, as TNF-α is detected in the inflamed exocrine glands. In certain autoimmune conditions, inhibition of TNF-α has been shown to be effective in suppressing tissue inflammation, and the use of anti-TNF agents has emerged as a potential therapeutic modality. Thus, many in vitro, in vivo, and even clinical trials have been conducted in the last 2 decades. However, the effect of TNF-α inhibitors on Sjogren's syndrome is unfortunately still equivocal. Recently, one study showed that the ineffectiveness was consistent with the absence of suppression of TNF-α.[19] Therefore, a more potent TNF-α inhibitor, which ameliorates the effects of free TNF-α, would be required to effectively suppress exocrine glandular inflammation to improve clinical signs and symptoms.

Non-Sjogren's type DE (NS-DE) represents a nonimmune type of dry eye disease, which is the most common form of

KCS and is caused by various conditions, including low humidity in the environment. Similar to Sjogren's syndrome, there have been many reports showing elevation of cytokines in ocular surface and tears in NS-DE.[9,10] Increased levels of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, have been detected in the tear fluid and conjunctival epithelium of patients with dry eye, as well as in dry eye mouse models.[20,21] However, anti-TNF antibody has not been used for treatment. Moreover, there have been no in vivo studies to evaluate the effects of anti-TNF-α in NS-DE, despite evidence of elevated TNF-α in this type of DE.

The purpose of this study was to develop potent TNF-α blockers to adequately suppress unbound ocular surface TNF-α and to investigate the effects of TNF-α inhibition on ocular surface and lacrimal gland inflammation using an in vivo controlled environmental chamber (CEC) mouse model.

Methods

Generation of HL036 From the TNFRI Gene by Site-Directed Mutagenesis

Human TNF receptor I (TNFRI) fragment (wild type) gene was synthesized in accordance with the sequence from amino acids 41 to 211 of the human *TNFRSF1A*, *P19438*. Amino acid substitutions of TNFRI fragments were generated by PCR-based site-directed mutagenesis. The PCR product of the *HL036* gene was inserted into the pET44a vector (Novagen, Darmstadt, Germany).

Expression and Purification of HL036

Wild type and HL036 were produced in inclusion body form in the *Escherichia coli* BL21Star (DE3) strain. The inclusion bodies were washed in 0.5% Triton X-100 and 5% glycerol, and solubilized in 50 mM Tris (pH 8.0), 2 mM EDTA, 1.75 M guanidine hydrochloride, and 0.2 M NaCl. Solubilized protein was refolded by dilution in a refolding buffer at 4°C. Refolded proteins were purified by size-exclusion chromatography using Superdex 75 (GE Healthcare Ltd., Piscataway, NJ).

Neutralizing Assay of TNF-α–Induced Cytotoxicity in Mouse WEHI-13 VAR Cells

Mouse WEHI-13VAR cells (American Type Culture Collections, Manassas, VA) were seeded into 96-well microtiter plates at a density of 2.0×10^4 cells/well in 20 mL RPMI 1640 (GIBCO, Grand Island, NY) media supplemented with 5% fetal bovine serum. The cells were further incubated at 37°C for 24 hours. Fifty microliters of 70 pg/mL TNF-α (R&D Systems, Minneapolis, MN), in the presence of 1.5 μg/mL actinomycin D (Sigma-Aldrich, St. Louis, MO) was added per well, and the cells were incubated with different concentrations of wild type (amino acids 41–211 of the human *TNFRSF1A*), HL036, and etanercept (Amgen, Thousand Oaks, CA), or medium control, for 24 hours at 37°C. Cell viability was measured by using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. All assays were performed in triplicate.

ELISA for Measuring TNF-α

After topical instillation of 0.25% HL036, the anterior and posterior halves of mouse eyeballs from eight eyes for each time point were collected, lysed, and subjected to total protein assay with a protein assay kit (Micro BCA; Pierce, Rockford, IL). The concentration of TNF-α in the cell lysates was determined by ELISA kits for mouse TNF-α (R&D Systems).

Surface Plasmon Resonance

The binding kinetics of the wild type, HL036, and etanercept for TNF-α were analyzed by surface plasmon resonance (SPR) (ProteOn XPR36; Bio-Rad, Hercules, CA). Samples were immobilized onto a GLC chip (Bio-Rad), which resulted in an increase of 1800 rat units (RU) (etanercept) or 250 RU (wild type and HL036). During the association phase, TNF-α diluted in PBS and Tween 20 (PBST) buffer (0.05% Tween-20, pH 7.4), at 50.0 nM, 25.0 nM, 12.5 nM, 6.25 nM, 3.125 nM, 1.563 nM, 0.781 nM, and 0.390 nM, was allowed to pass over the chips immobilized with wild type, HL036, and etanercept at a flow rate of 30 mL/min for 4 minutes. During the dissociation phase, PBST buffer was applied to the sensor chip at a flow rate of 30 mL/min for 10 minutes. The data were analyzed globally with the ProteOn Manager software (Bio-Rad), using a 1:1 Langmuir binding model.

Animals and Dry Eye Induction

Six- to 8-week-old female (C57BL/6) mice (Charles River Laboratory, Wilmington, MA) were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by Institutional Animal Care and Use Committee of the Yonsei University College of Medicine. Dry eye was induced in the mice by placing them in a CEC as described previously.[22] Six mice were allocated to each treatment group. To achieve maximum ocular surface dryness, the mice in the CEC received subcutaneous injections of 0.1 mL scopolamine hydrobromide (5 mg/mL; Sigma-Aldrich), three times a day, for the duration of the experiment. Dry eye induction and tissue preparation were performed according to a previous protocol.[22] As each experiment was repeated three times, calculating the average values, more than 12 biological replicates were used at each time point to determine the expression data.

Treatment Regimen and Tissue Preparations

After 10 to 14 days of dry eye induction, confirming grade 3 corneal erosions or above, the mice were divided into vehicle (sodium acetate, pH = 5.5), etanercept, and TNF-α blocker HL036 treatment groups. Treatment was given for 1 week. To investigate the effect of TNF-α blockers, HL036, and etanercept, two different concentrations of each substance (0.25 and 2.5 mg/mL) were administered subcutaneously (0.1 mL, twice a day) or topically (8 μL, three times a day) for 1 week, into eyeballs of dry eye–induced mice.

Mice were killed, and lids, eyeballs, and lacrimal glands were collected. Each of the tissues was halved, and one-half was fixed in 3.7% paraformaldehyde and stored until immunostained. The other half was stored at −70°C for quantitative RT-PCR (qRT-PCR).

Corneal Erosion Scoring of the Mouse

The corneal erosion grading used in this study was as has been described previously.[23] Briefly, at the end of treatment, 1 mL 1% fluorescein (Sigma-Aldrich) was applied to the lower conjunctival sac of the mice, and after 3 minutes, corneal fluorescein staining was examined with a slit lamp biomicroscope. Punctate staining was evaluated in a masked fashion using the Oxford Scheme grading system, giving a grade from 0 to 4 for each cornea.

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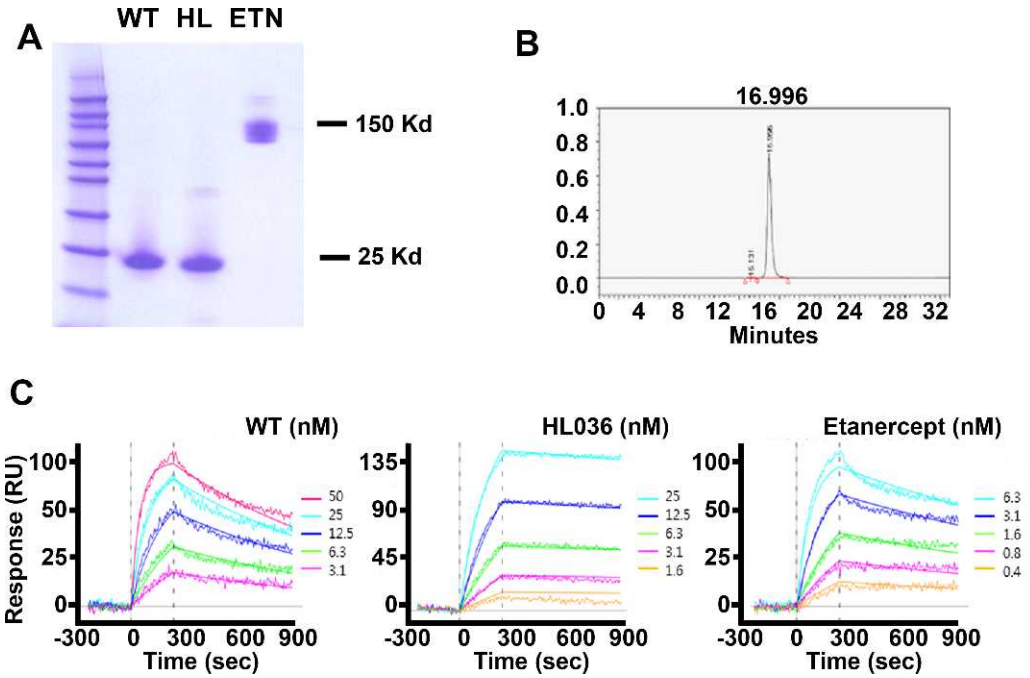

FIGURE 1. Physical properties and characteristics of TNF-α blocker HL036. (**A**) A total of 0.5 μg purified HL036 and etanercept were resolved on 12% SDS-PAGE and stained with Coomassie Brilliant Blue (from left to right: protein marker, WT, HL, etanercept [ETN]). (**B**) HPLC of HL036, with 0.5 μg HL036 loaded onto a SE-HPLC column to yield the resolved protein data. (**C**) SPR data of WT, HL036, and ETN-treated samples. Purified proteins were allowed to flow over SPR chips bearing immobilized TNF-α at a concentration of 500 nM. All data were corrected for unbound chip background.

PAS and Immunohistochemical Staining

Central vertical plane sections of 6-μm thickness were stained with hematoxylin-PAS counterstaining. Briefly, the slide was exposed periodically to acid solution for 15 minutes. Then, after rinsing with tap water, Schiff's reagent was exposed for 15 minutes, and counterstaining with hematoxylin was performed. One masked observer counted the number of goblet cells in the superior or inferior conjunctiva. Goblet cell counting was performed under a microscope using a ×10 objective. Five different sections through each segment (inferior and superior) were selected for counting, and the averages were calculated. This method was modified from previous work.[22] The immunostaining method for lacrimal glands has been described previously.[24] CD3 (Rabbit monoclonal, 1:200; Lab Vision Corp., Fremont, CA) was used for immunochemical staining.

Tissue RNA Extraction and qRT-PCR

Four to six corneas or six lacrimal glands from four to six mice were included in each group. RNA was isolated with RNeasy Micro Kit (QIAGEN, Valencia, CA) from mouse full-thickness cornea and lacrimal glands, and reverse transcribed using Superscript III Kit (Invitrogen, Carlsbad, CA). Real-time qPCR was performed using TaqMan Universal PCR Mastermix and preformulated primers for IFN-γ (Mm01168134_m1; Applied Biosystems, Foster City, CA), IL-6 (Mm00446190_m1), IL-12 (Mm00434165_m1), TNF-α (Mm004443260_g1), IL-1β (Mm00434228_m1), and GAPDH (Mm99999915_g1).

TABLE. The Binding Kinetic Profiles of the Wild Type, HL036, and Etanercept to TNF-α, Obtained by SPR

Sample	K_a* ($M^{-1}s^{-1}$)	K_d† (s^{-1})	K_D‡ (M)
Wild type	7.89×10^5	1.04×10^{-3}	3.26×10^{-9}
HL036	3.12×10^5	9.10×10^{-5}	3.04×10^{-10}
Etanercept	1.79×10^6	4.35×10^{-4}	2.42×10^{-10}

The SPR data were analyzed by ProteOn Manager Software.
* Association kinetic constant.
† Dissociation kinetic constant.
‡ Equilibrium dissociation constant.

Luminex Assay for Measuring Cytokines Concentration

Five dry eyes and normal corneas were pooled per sample for assay. In both dry eye and normal condition, the results were reported as the amount of protein per group. Corneas obtained from each mouse were quartered in sterile 1× PBS, and then placed in 300 μL + complete protease inhibitor (Complex Mini Protease Inhibitor; Roche Applied Science, Indianapolis, IN). Then, samples were sonicated (Sonic Dismembrator, Model 100; Fisher Scientific, Pittsburgh, PA) for 25 seconds, and the sonicator tip was rinsed with 100 μL PBS + protease inhibitor, yielding a final volume of 800 μL/sample. Samples were microcentrifuged to remove cellular debris. The cytokines were assayed with a mouse cytokine/chemokine kit from Millipore (Multiplex, MPXMCYTO-70K; Millipore Corp., St. Charles, MO) on a Luminex 100 (Bio-Rad) according to the manufacturer's instructions. Standard curves were generated by using the reference cytokine concentrations supplied by the manufacturer. Raw data (mean fluorescent intensity) were analyzed by commercially available software (Bio-Plex Manager Software; Bio-Rad) and converted into concentration values. The total protein concentration of each corneal sample was measured using the micro-bovine serum albumin method to calculate the ratio of cytokine to total corneal protein.[25,26]

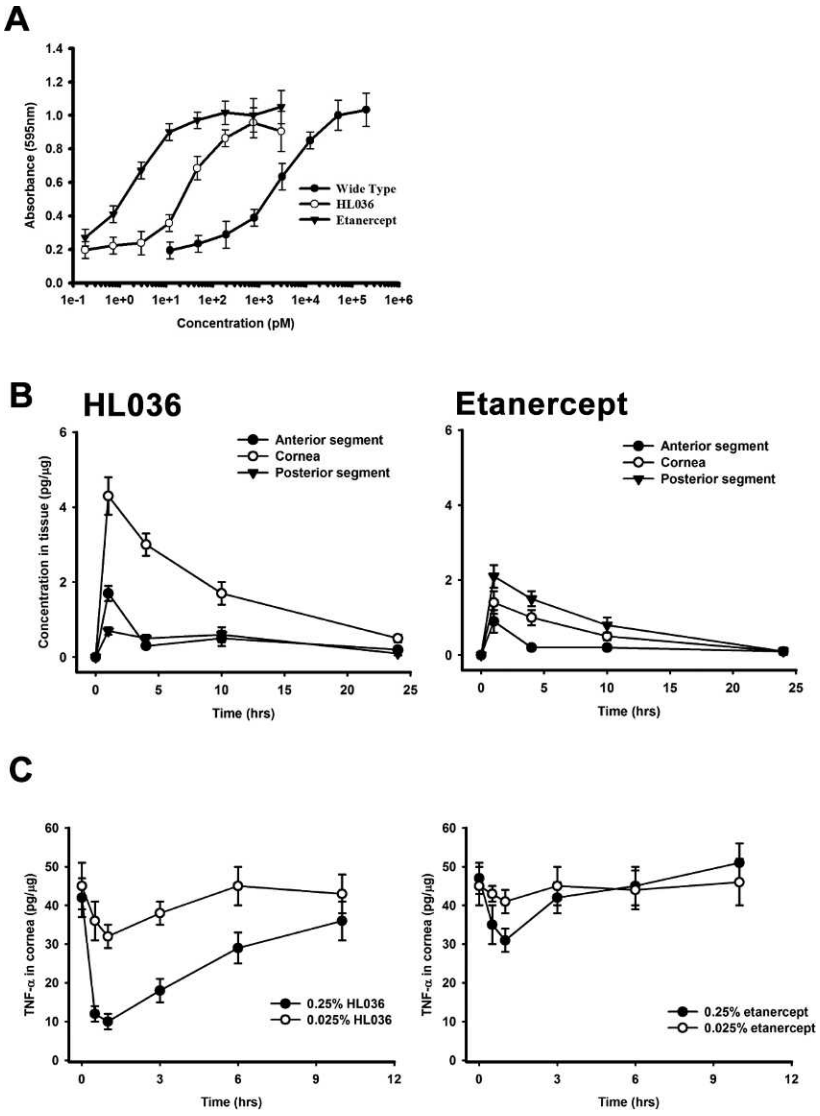

FIGURE 2. Determination of EC_{50} values and tissue distributions of HL036 in a dry eye model. (**A**) WEHI cells were treated with 1.5 μg/mL actinomycin D with 50 μL human TNF-α (70 pg/mL), and simultaneous serial dilutions of the three proteins were added. All cells were then incubated for 24 hours. Thereafter, the inhibition of cytotoxicity was detected by MTT assay. Colorimetric values of cell death were measured and calculated for eight different concentration points. (**B**) Concentrations of HL036 were measured in the anterior segment of eyeball tissues, cornea, and posterior segment. HL036 and etanercept (5 μg/20 μL) was topically administered. Six eyes at each time point were taken and each tissue was separated. HL036 concentration was measured by HPLC. (**C**) TNF-α in the unbound form was measured by ELISA in the anterior segments of eyeballs after 3 days administration of HL036 or etanercept to induce dry eye in mouse cornea.

Statistical Analysis

The Mann-Whitney *U* test or the *t*-test for independent samples was performed to compare differences between the two groups. One-way ANOVA was used to make comparisons among three or more groups, and Dunnett's test was further used to compare each treated group with the control group. A P value < 0.05 was considered to indicate a significant difference.

RESULTS

Physical Properties and Characterizations of the TNF-α Blocker HL036

The designed novel proteins were successfully produced by following the methods described above. The "wild type (WT)," an original TNF-α binding segment of TNFR1, and HL036 (HL), the peptide designed with several amino acid substitutions from WT, were expressed as 19-kD single bands on SDS-PAGE (Fig. 1A). To further show purity and molecular weights for the proteins, size exclusion (SE)–HPCL was used, and the molecular weight of HL036 was calculated as 16.966 g/mol and was over 90% pure (Fig. 1B).

To delineate the binding activity of TNF-α and synthetic peptide, SRP analysis was used (Fig. 1C, Table). Of interest, compared with WT, the equilibrium dissociation constant (K_D) of HL036 for TNF-α showed a 10.7-fold decrease, which meant that the affinity of HL036 for TNF-α was significantly enhanced by amino acid substitution. In addition, the binding kinetics data indicated that the ligand-binding mode of HL036 might differ from that of WT and etanercept. Specifically, the dissociation kinetic constant (K_d) of HL036 for TNF-α (9.10×10^{-5} s^{-1}) was markedly lower than that of WT (1.04×10^{-3} s^{-1}) and etanercept (4.35×10^{-4} s^{-1}), which meant tighter binding and less dissociated free TNF-α. The association kinetic constant (K_a) of HL036 (3.12×10^5 M^{-1} s^{-1}) for TNF-α was

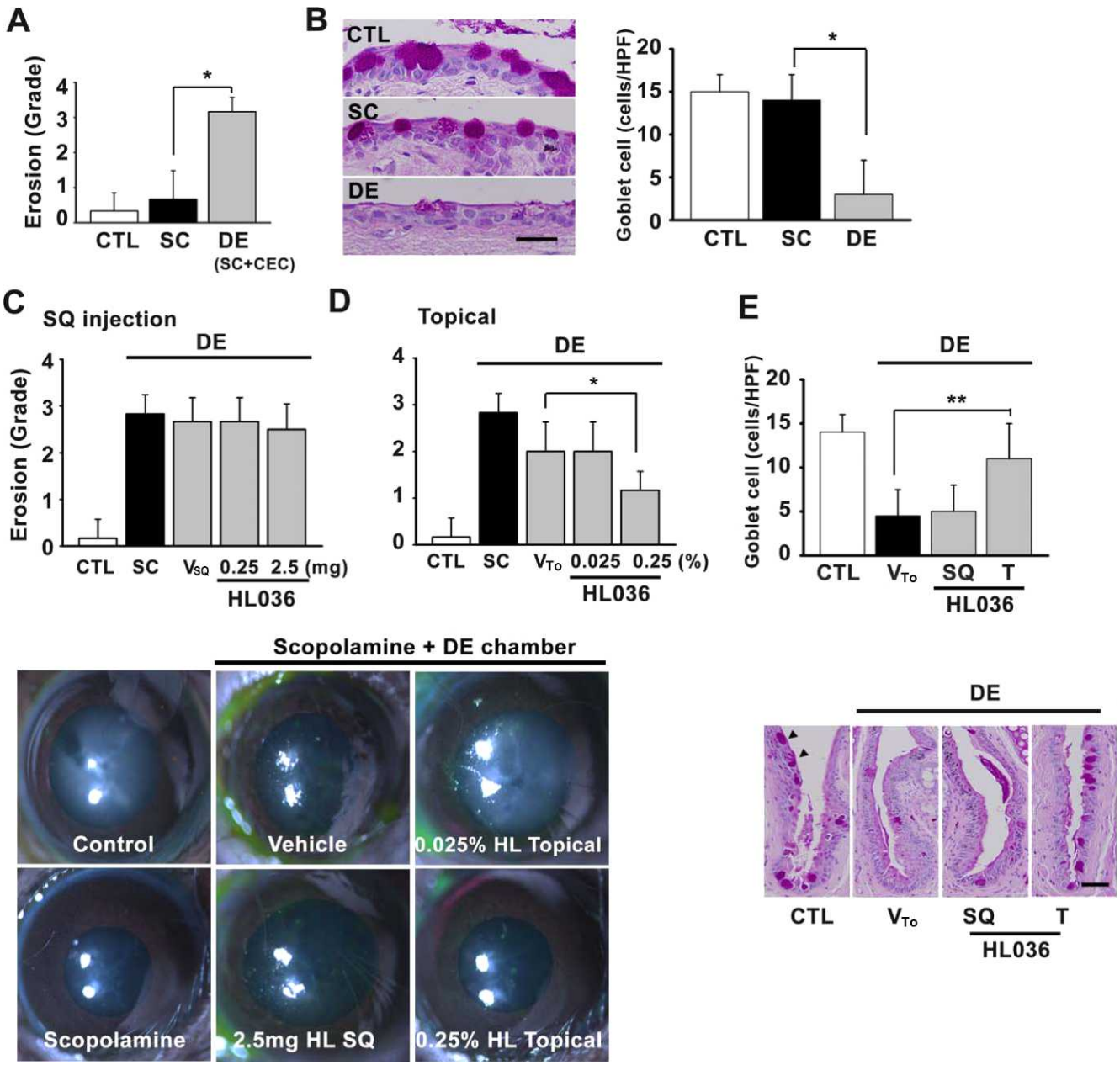

FIGURE 3. Determination of clinical data and histologic findings of ocular surface with HL036. (**A**) Corneal erosion and (**B**) goblet cell density were measured, followed by fluorescein cornea surface staining. Each group included four to six corneas randomly selected from both eyes. Goblet cell density was measured at 14 days after each treatment and calculated from five independent microscopic fields ($^{*}P < 0.05$, $^{**}P < 0.01$ compared between SC and DE; *black scale bar*: 20 μm). (**C**, **D**) Corneal erosion was measured in dry eye–induced mouse after a week of treatment with subcutaneous injection (**C**) or topical application (**D**) of HL036. Representative photographs from each group are shown. (**E**) Goblet cells were counted in DE after HL036 treatment conditions. The cells were counted at magnification ×100 in five random sections, and calculated mean values were obtained. $^{**}P < 0.01$ compared with DE condition; *black scale bar*: 50 μm. *Arrowhead* indicates goblet cells. CTL, control; DE, scopolamine injection with environmental chamber induction; SC, scopolamine injection without environmental chamber induction; SQ, 2.5 mg HL036 subcutaneous injection; T, 0.25% HL036 topical administration; V_{SQ}, subcutaneous vesicle injection; V_{To}, topical vesicle.

only slightly higher than that of WT (7.89×10^5 M^{-1} s^{-1}) and lower than that of etanercept (1.79×10^6 M^{-1} s^{-1}). The Table summarizes the SPR results.

Determination of Half Maximal Effective Concentration (EC_{50}) and Tissue Distribution of HL036 in a Dry Eye Model

We determined the neutralizing activity of HL036 and compared the activity with WT and etanercept by cytotoxicity assay using TNF-α sensitive WEHI-13 VAR cells. As shown in Fig. 2A, HL036 exhibited approximately a 160-fold elevation of neutralizing activity for TNF-α compared with the EC_{50} of WT (2790 pmol), before amino acid substitution. However, the EC_{50} value of HL036 (17.3 pmol) was still 4.8 times higher than that of etanercept (3.6 pmol).

The above results implied that HL036 had improved dissociation kinetics and TNF-α binding ability before amino acid substitution. Therefore, the biologic effects were investigated and compared with the blockers using an in vivo mouse

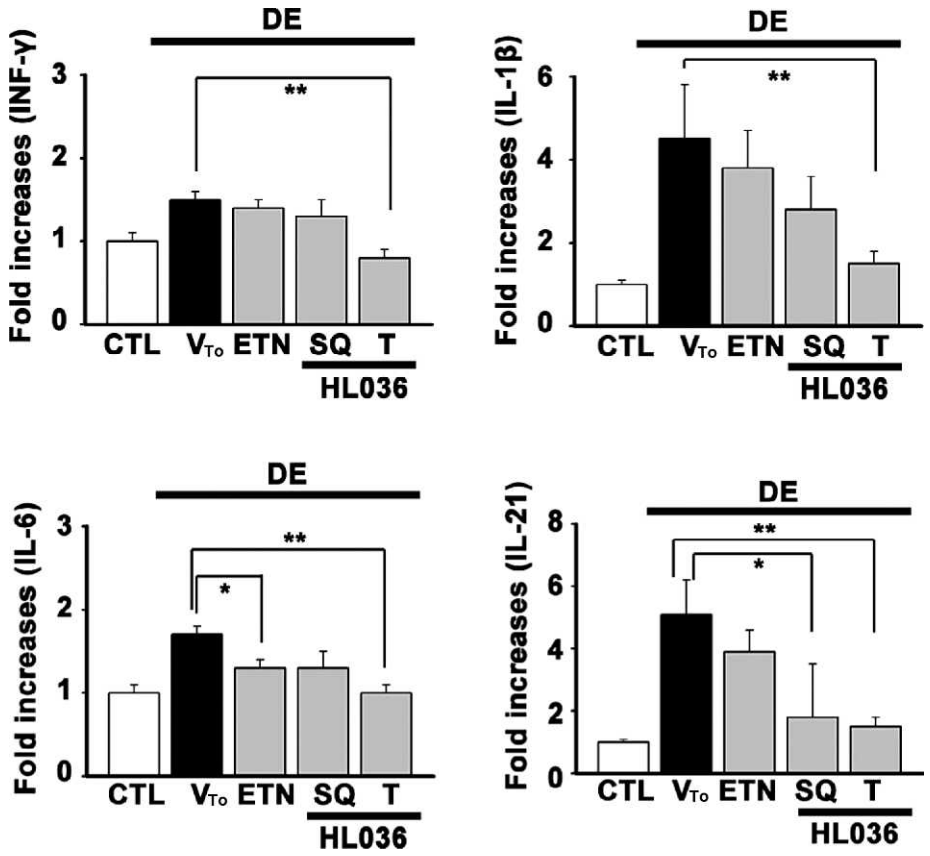

FIGURE 4. The mRNA expression of proinflammatory cytokines in dry eye after topical administration of 0.25% HL036. The mRNA level of each cytokine was measured in mouse corneal tissues after 7 days of treatment with each substance in DE-induced B6 mice. Four to six corneas from each treatment group were used for qRT-PCR compared with non–dry eye control (CTL) mouse corneas. $^{*}P < 0.05$, $^{**}P < 0.01$.

dry eye model. The tissue distribution and concentration of the blockers were measured. After systemic administration, neither HL036 nor etanercept was detected in cornea.

With topical administration, HL036 showed significantly higher corneal tissue concentration than etanercept (Fig. 2B). The corneal concentration of HL036 was 2.3 times higher than etanercept 1 hour after corneal instillation, and continuously higher tissue concentration was observed until 24 hours. Moreover, the free levels of TNF-α were also significantly lowered by treatment with HL036 (Fig. 2C) when compared with WT or etanercept. However, in the posterior segment, etanercept concentration was much higher than HL036.

Corneal Erosion Score, Goblet Cell Counts, and Stromal Cell Infiltration After HL036 Treatment

HL036 was applied in vivo in the mouse dry eye model and pathologic changes in ocular surfaces were determined. At first, we compared corneal erosion and goblet cell density between scopolamine injection only and scopolamine with a CEC (Figs. 3A, 3B). After 14 days of induction, using scopolamine peritoneal injection with CEC caused most significant corneal erosion; all the rest of the experiments were performed using both a low humidity CEC and scopolamine injection. Among the different administration conditions, corneal erosion grade were significantly improved with topical application of 0.25% HL036, compared with vehicle (Figs. 3C, 3D). The mean corneal erosion grade was 2.8 ± 0.9 after induction of dry eye (SC, scopolamine injection without environmental chamber induction; Fig. 3C). After treatment with topical 0.25% HL036, mean erosion grade was decreased to 1.4 ± 0.4 ($P < 0.001$, *t*-test). However, erosion score for neither vehicle nor HL036 injection was changed (Fig. 3C). With the low concentration topical application of HL037 (0.025%), the erosion score decreased slightly but did not show a significant difference from vehicle treatment (Fig. 3D).

The goblet cell count after different administration routes of HL036 was determined. After topical application of 0.25% HL036, goblet cell count was increased, and the shape of the goblet cells were normal (Fig. 3E). However, vehicle or subcutaneous injection of 2.5mg HL036 did not improve the goblet cell loss from DE stimuli.

Effects of HL036 on Proinflammatory Cytokine Expression in Dry Eye, After Different Administration Routes

Using qPCR analysis, elevations of inflammatory cytokines and chemokines were observed in DE conditions. IFN-γ, IL-1β, IL-6, and IL-21mRNA levels were elevated in dry eye cornea (Fig. 4). Only topical administration of 0.25% HL036, not subcutaneous administration, nor etanercept, produced decreased inflammatory cytokine levels in cornea. Among these cytokines, the levels of IL-1β and IL-21 were much higher than that of IFN-γ and IL-6. As previously reported in a human study,[19] etanercept did not produce adequate downregulation of inflammatory cytokines in ocular surfaces.

Protein levels of inflammatory cytokines in ocular surfaces were measured by the multiplex bead method. Similar to mRNA levels measured by qPCR, in the DE condition IFN-γ, IL-6, and IL-21 protein levels increased significantly (Fig. 5). The

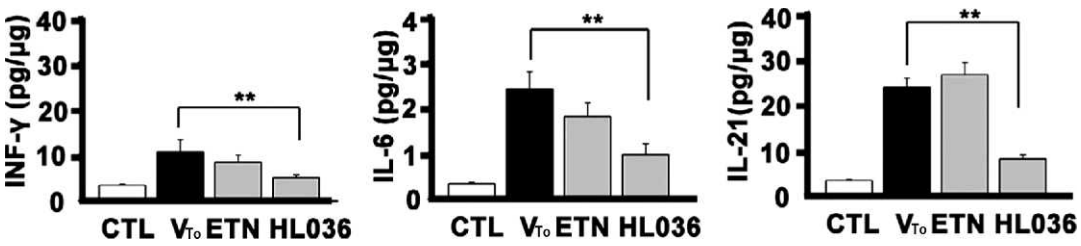

FIGURE 5. Chemokine Luminex assay for IFN-γ, IL-6, and IL-21 in dry eye. (**A**) Cornea and cornea and limbal tissues were prepared, and multiplex cytokine and chemokine Luminex assay was performed according to the manufacturer's protocol. All experiments were performed with four to six corneas from each treatment group. Each measured chemokine concentration (pg/mL) was divided by total protein concentration (μg/mL) of each sample. ETN, topical 0.25% etanercept; HL036, topical 0.25% HL036 administration. $^{**}P < 0.01$ compared with topical vesicle group (V_{To}).

elevated cytokines were downregulated by topical HL036. Cytokine concentrations were not changed by treatment with etanercept.

Change of Inflammatory Cytokines in Lacrimal Gland by Treatment With TNF Blockers and Anti-Inflammatory Drugs

Because the ocular surface and lacrimal gland are closely connected by neural networks, in pathophysiological progression of DE,[27] we measured cytokine levels in lacrimal gland and compared them with those of cornea. Of interest, IFN-γ and IL-6 were found to be increased during DE. Moreover, the levels were more increased in lacrimal gland than ocular surfaces in DE. However, the level of elevation of lacrimal gland IL-1β was similar to that of ocular surfaces and did not show significant differences. IL-21 was more elevated in cornea than in lacrimal gland (Fig. 6A). With topical administration of HL036, expression of lacrimal gland IFN-γ and IL-6 mRNA was significantly decreased to non-DE control levels (Fig. 6B). However, the same concentration of etanercept did not suppress IFN-γ mRNA expression and slightly decreased the expression of IL-6 mRNA in DE-induced lacrimal gland (Fig. 6B).

Then, DE-induced T-cell infiltration was measured by counting $CD3^+$ cells (Fig. 6D). Compared with the control, DE with vehicle treatment showed many infiltrating cells in lacrimal glands, including $CD3^+$ cells (red arrow). The infiltrated $CD3^+$ cells were significantly decreased by topical application of 0.25% HL036. Last, we additionally measured infiltration of other types of inflammatory cells to lacrimal glands. Of interest, $CD11b^+$ and $CD11b^+Gr1^+$ were found to be significantly increased in the DE condition (Fig. 6E). In controls, approximately 1.0% of cells were found to exhibit $CD11b^+$. In dry eyes, this increased to 4.3%. Thereafter, topical treatment of 0.25% HL036 inhibited lacrimal gland $CD11b^+$ and $CD11b^+Gr1^+$ cells, returning these cell fractions almost to their levels in the normal condition.

DISCUSSION

We have developed a novel TNF-α blocking peptide by modifying the TNF-α binding region of the TNF-α receptor. The blocking peptide was effective in blocking TNF-α activities, which resulted in suppressed cytokine production in ocular surfaces as well as in lacrimal glands in the in vivo mouse DE model. Moreover, by neutralization of ocular surface free TNF-α, the DE-induced cytokine increase in lacrimal gland was downregulated, and healthy glandular morphology was maintained.

Previous studies have shown that TNF-α blocking is the most effective means of suppressing inflammation for many pathologic conditions, including DE.[12,28,29] Therefore, it is not unusual that many clinical or experimental trials have been performed to treat DE with TNF-α blocking agents. However, a larger randomized, double-blind, placebo-controlled study of infliximab with Sjogren's patients showed only partial efficacy in severe Sjogren's patients, and no difference in improvement of dryness and other systemic symptoms between the placebo- and the TNF-α–treated group.[16] Similarly, etanercept treatments also failed to improve clinical results above placebo levels in placebo-controlled studies.[19,30,31] These studies did not provide an explanation for the poor results. In a clinical trial with etanercept, a paradox was noted with raised circulating levels of TNF-α. Moutsopoulos et al. reported that etanercept not only bound to and neutralized TNF-α but also stabilized and prolonged its half-life.[19] Therefore, a neutralizing TNF-α blocker with higher affinity, not a temporally binding TNF-α blocker, might be required to achieve positive results from TNF-α blocking treatment.

HL036 has been developed and selected from many candidate molecules that were differentially designed with substituted amino acid sequences of TNF-α receptors to optimize TNF-α binding efficiency. To determine the TNF-α binding affinity of the above candidate molecules, the TNF-α binding efficiency was measured by SPR. Several candidates that showed strong binding effectiveness with low K_d values were studied. HL036 was finally selected based on the results of in vivo experiments for improving corneal inflammation and reducing cytokine induction (data not shown). Because the HL036 was conceptually similar to etanercept, we compared TNF-α neutralizing biological activities with etanercept, not with infliximab or others. The anti-inflammatory effect of cytokine induction and clinical improvement by HL036 was greater than the effect observed by etanercept. The improved clinical and in vivo experimental results from HL036 versus etanercept were not fully investigated in this study. However, HL036 showed improved dissociation constants (K_d value) when compared with etanercept as shown by SPR. This suggests that the TNF-α is more tightly bound, resulting in decreased free TNF-α concentration in ocular tissues. In addition, HL036 showed improved tissue penetration and higher tissue concentration in cornea, which in turn improved the effectiveness of its TNF-α binding abilities over etanercept on the ocular surface. However, etanercept mainly accumulated in the posterior segment. Based on our results, HL036 is more suitable for ocular surface inflammation, such as DE, than etanercept.

We also showed that the drug delivery route is important to achieve adequate suppression of target organ inflammation. In other studies, TNF-α blockers were applied systemically. However, these studies did not measure the concentration of blockers and TNF-α in target organs, such as lacrimal gland and salivary glands. When we compared the concentrations of TNF-α blocker and TNF-α in lacrimal gland and cornea with the

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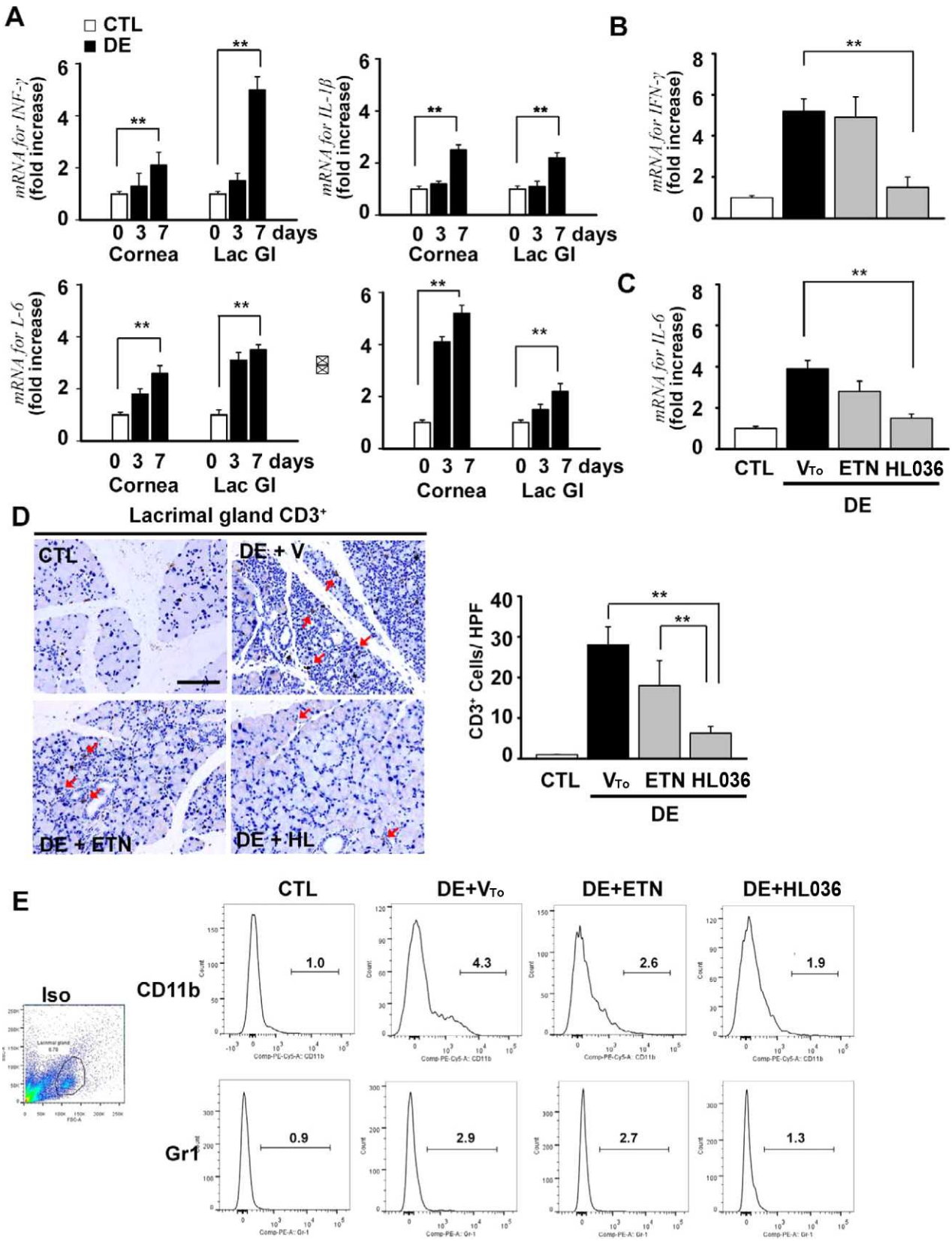

Figure 6. Change of inflammatory cytokines in lacrimal gland after treatment with TNF blockers. (**A**) The mRNA level of each cytokine was measured in cornea and lacrimal gland (Lac GL) after 3 and 7 days of DE induction in B6 mice. Four to six corneas and six lacrimal glands from each treatment group were obtained, and qRT-PCR was performed for each cytokine and compared with non-dry eye control mouse cornea (**$P < 0.01$). (**B**) IFN-γ and (**C**) IL-6 mRNA levels were measured after 7 days of topical treatment. HL036, topical 0.25% HL036. (**$P < 0.01$, compared with V_{To}). (**D**) Immunohistochemical staining of $CD3^+$ cells in lacrimal glands. Lacrimal glands from dry eyes induced in mice were secured after 7 days of topical treatment. $CD3^+$ cells were counted from five independent high power fields (HPF) for each treatment group (*red arrow*, $CD3^+$ cells; **$P <$

0.01; *black scale bar*: 20 μm) (**E**) Six lacrimal glands from each treatment group were collected and digested with collagenase/DNase 1. Cells (1×10^6/sample) were stained with PE-Cy5–labeled CD11b and PE-labeled Gr1 and analyzed by flow cytometry. Fractions of stained cells were calculated and presented.

administration route, the concentration of HL036 was much higher in ocular surfaces after topical application as compared with subcutaneous administration (Fig. 2B). The drug was detected within several minutes after topical administration, and elevated concentration was continuous until 30 minutes, then slowly decreased after 12 hours of treatment. However, using the subcutaneous route, both HL036 and etanercept were not detected on ocular surfaces.

We found elevation in cytokines by DE induction, which was downregulated by HL036 treatment. Among them, IL-21 was unusually increased in DE-induced mice. Previously, IL-21 was shown to be expressed in activated $CD4^+$ cells, especially Th2 and Th17 subsets.[14,32] Of interest, previous reports have documented IL-21 upregulation in Sjogren's syndrome,[33,34] as well as the upregulation of Th1 and Th17 response gene expression.[7,35] However, studies have yet to clearly explain why desiccation stress or other DE models induce Th1 and Th17 responses. Moreover, although Th1 and 17 responses usually arise from specific immune responses, the immune-related substances in dry eyes remain unknown. IL-21 elevation as it relates to cytokine elevation by DE should be addressed in the future.

In lacrimal gland, the topically administrated HL036 and etanercept were not detected, so the HL036 may not have a direct effect on this tissue. The inflammatory condition of lacrimal glands was significantly improved only by the topical route. Although we cannot fully explain these results, the effect of topical HL036 treatment on lacrimal glands might be by an indirect effect, through suppression of ocular surface inflammation. As the lacrimal gland and ocular surface are closely connected by a neural network, the reduced inflammatory condition in ocular surface after topical HL036 may also alter inflammatory signals to the lacrimal gland. This route may be more effective than other modes of HL036 administration. In non-disease tissues, these inflammatory cytokine concentrations were not changed by HL036 treatment through both administration routes (data not shown). Therefore, to reduce lacrimal gland inflammation in DE, the downregulation of inflammatory conditions in ocular surfaces is essential, and more effective drugs to block the neural network between ocular surfaces and lacrimal glands are needed to treat DE.

Limitations of the study include the use of the mouse environmental chamber model for a short period of only 3 to 4 weeks. As DE is a long-term disease, the pathologic changes in target organs such as the lacrimal gland may not be observed in a short study. Thus, the results may not reflect the actual human condition, which has an onset of at least several months. Because of the limitations of the in vivo model used, we measured only the changes after several weeks of treatment. The activation of compensatory mechanisms, as seen in the human disease, would not be observed in this short-term study. A longer follow-up to measure changes in cytokine levels is needed to address the exact mechanism of DE. Because most TNF-α blockers were investigated in human trials of Sjogren's disease, the effects of HL036 must be determined by human trials of dry eye disease. Last, infliximab, another type of TNF-α blocker, has been reported to be effective in treating dry eyes.[36] Therefore, the effect of HL036 on DE should be compared with that of infliximab.

In spite of previous equivocal results of TNF-α treatment for Sjogren's type dry eye, it is still apparent that TNF-α plays an important role in the pathogenesis of many inflammatory and immunologic diseases, including dry eye. We provide evidence of improved ocular surface and lacrimal gland inflammation through sufficient neutralization of TNF-α. Therefore, we suggest that by using appropriate administration routes for the drugs, adequate inhibition of TNF-α will result in improvement of DE or even Sjogren's type syndrome. Future human trials are needed to verify our in vivo results and may help to further clarify the role of TNF-α in the pathophysiologic disease mechanisms of DE.

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