Cornea

HIF1α-mediated TRAIL Expression Regulates Lacrimal Gland Inflammation in Dry Eye Disease

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Purpose. The purpose of this study was to investigate the expression of death ligands in the lacrimal glands (LGs), identify upstream factors that regulate their expression, and determine the functional roles of these factors in the pathogenesis of dry eye disease (DED).

Methods. For DED experiment, ex vivo coculture system with LG and in vivo murine model using a controlled environment chamber were utilized. C57BL/6 mice and hypoxia-inducible factor (HIF)-1α conditional knockout (CKO) mice were used. Immunohistochemical staining, polymerase chain reaction, and immunoblotting were performed to determine levels of death ligands including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in DED-induced LGs. Additionally, acinar cell and $CD45^+$ cell apoptosis was determined with neutralizing TRAIL treatment.

Results. Desiccating stress significantly increased HIF-1α expression in LG-acinar cells. Furthermore, HIF-1α deficiency significantly enhanced the infiltration of $CD45^+$ inflammatory cells in LG and induced LG-acinar cell death. Meanwhile, only TRAIL expression was increased in DED-LG, but abrogated in HIF-1α CKO. Interestingly, the main source of TRAIL was the $CD45^-$ LG-acinar cells, but not $CD45^+$ immune cells after DED induction. Using ex vivo coculture system, we confirmed LG-induced apoptosis of immune cells via HIF-1α-mediated TRAIL secretion following DED. Consistent with ex vivo, the insufficiency of HIF-1α and TRAIL enhanced recruitment of inflammatory cells to the LG and subsequently exacerbated ocular surface damage in DED mice.

Conclusions. Our findings offer novel insight into the regulatory function of acinar cell-derived TRAIL in limiting inflammatory damage and could be implicated in the development of potential therapeutic strategies for DED.

Keywords: death ligand, tumor necrosis factor-related apoptosis-inducing ligand, hypoxia-inducible factor-1α, lacrimal gland, dry eye disease

Dry eye disease (DED) is characterized by dysfunction of the lacrimal functional unit, composed of the lacrimal gland (LG), ocular surface, and connecting sensory nerves.[1] Impaired function of the LG leads to reduced tear formation and propagates damage to the ocular surface. Moreover, etiological factors, including desiccating stress, induce ocular surface damage by initiating an inflammatory cascade.[2] Despite multiple reports demonstrating the major contribution of excessive inflammation in the pathogenesis of Sjögren and non-Sjögren DED, the mechanism by which LG regulates the infiltration of inflammatory cells during desiccating stress has not been fully understood.

Our previous report has shown that hypoxia-inducible factor (HIF)-1α, an oxygen-dependent transcriptional factor,[3,4] prevents LG acinar cell damage in DED. Moreover, LG-specific deletion of HIF-1α positively correlated with increased infiltration of inflammatory cells during DED. Although our previous reports elucidate the association of HIF-1α with LG inflammation, the underlying mechanism and immunoregulatory functions of HIF-1α in LG during DED remains unknown.

HIF-1α has been associated with elevated expression of death ligands,[5] which have recently been shown to regulate inflammation by inducing cell death in cancer[6,7] and autoimmune disorders.[8] As a type II integral membrane protein of the tumor necrosis factor (TNF) family, TNF-related apoptosis-inducing ligand (TRAIL) has been reported to induce apoptosis of tumor cells.[9] Interaction of TRAIL

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with its receptor, particularly death receptor 5 (DR5), promotes apoptosis by activating the caspase-dependent pathways.[10,11] DcR1, a decoy receptor, lacks a signaling domain and inhibits the TRAIL-induced apoptosis by competing with DR5.[9,10,12] Although the elevated tissue levels of TRAIL have been observed in a variety of inflammatory conditions,[13,14] the contribution of TRAIL in regulating desiccation-induced ocular inflammation has not been investigated.

The current study investigates the expression levels and immunoregulatory function of death ligands in a mouse model of DED. Our data demonstrate that acinar cell–expressed HIF-1α upregulates the secretion of TRAIL, which in turn limits inflammation of LG and progression of ocular surface epitheliopathy by inducing apoptosis of infiltrating leukocytes in DED.

Materials and Methods

Animals, DED Induction, and Generation of HIF-1α Conditional Knockout Model

Six- to 8-week-old male C57BL/6 mice (Charles River Laboratory, Wilmington, MA) were used in accordance with the standards set forth in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (YU-2015-0190).

DED was induced by placing the mice in a controlled environment chamber (CEC). To achieve maximum ocular surface dryness, mice in the CEC (with relative humidity below 13%) were given subcutaneous injections of 0.1 ml scopolamine hydrobromide (5 mg/ml; Sigma-Aldrich Chemical Co., St. Louis, MO) three times a day.

The detailed methods for generating and breeding HIF-1α conditional knockout (CKO) mice have been described previously.[15] Concisely, mice with MMTV-Cre-dependent knockout at the *hif1α* locus were generated by crossing animals containing loxP sites flanking exon 2 of *hif1α* (B6.129-$Hif1\alpha^{tm3Rsjo}$/J; Jackson Laboratory, Bar Harbor, ME). Genotyping was performed using DNA from tail biopsies. All experiments were performed on littermates derived from Hif1αf/f-MMTV-Cre^{+} × Hif1αf/f-MMTV-Cre^{-} (6-10 weeks old).

Tissue Preparation

After DED induction, mice were sacrificed and LGs were harvested on day 7 and day 10. Each tissue sample was either fixed in 3.7% paraformaldehyde for immunostaining or stored at -70°C for real-time quantitative polymerase chain reaction (qPCR) or immunoblotting.

RNA Isolation and Real-Time qPCR

RNA was isolated from the mouse LGs using a RNeasy Micro Kit (QIAGEN, Valencia, CA), and reverse transcription was performed using a Superscript III Kit (Invitrogen, Carlsbad, CA). Real-time qPCR was performed using SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) with a StepOnePlus real-time qPCR System (Applied Biosystems, Foster City, CA). Preformulated primers for TRAIL (Mm01283606_m1), HIF-1α (Mm00468869_m1), and glyceraldehyde-3-phosphate dehydrogenase (Mm_001289726_m1) were sourced from Applied Biosystems (Carlsbad, CA) to evaluate messenger RNA (mRNA) expression. The mRNA primer sequences for DcR1, programmed death ligand-1 (PDL-1), and FasL were custom designed as described in Supplementary Table S1. Gapdh served as an internal control, and gene expression was presented as fold change from the control. The analysis was performed using the comparative threshold cycle method.

Immunohistochemical Staining

The immunohistochemical (IHC) staining method for LGs has been described previously.[15] Briefly, LGs were harvested, and histologic sections (5-7 μm) were collected on poly-L-lysine-coated slides and deparaffinized. Sections were blocked with rabbit, goat, or rat serum for 40 min at room temperature and exposed to primary antibodies: TRAIL (Abcam Inc., UK), CD11b (Abcam Inc.) and HIF-1α (Abcam Inc.). Antibodies were diluted 1:100 to 1:200 and incubated overnight at 4°C. After washing in Tris-buffered saline supplemented with Tween 20, each section was exposed to secondary antibodies for 1 hour. After washing with Tris-buffered saline supplemented with Tween 20, the sections were exposed to 4',6-diamidino-2-phenylindole (PureBlu, Bio-Rad, Inc., Hercules, CA). Light microscopy (Axio Imager 2, Carl Zeiss, Germany) was used to examine the samples.

Immunoblotting

Total protein concentrations of supernatant fractions were determined using the bicinchoninic acid (BCA) protein assay (Bio-Rad, Inc.). Equal amounts of protein aliquots were boiled in equal volumes of 2 × SDS Laemmli sample buffer and resolved by 8% or 10% (wt/vol) with primary antibodies: anti-TRAIL (0.2 μg/mL; R&D System Inc., Minneapolis, MN), anti-PDL-1 (0.1 μg/ml; Cell Signaling Inc.), anti-DR5 (1 μg/mL; R&D System Inc.), anti-mDcR1 (1 μg/mL; R&D System Inc.), and anti-β-actin (2 μg/ml; Abcam Inc., UK). β-actin was used as an internal loading control.

Terminal Deoxynucleotidyl TUNEL Staining

LGs were fixed in 10% buffered formalin. Sections (7 μm) were cut, fixed, deparaffinized, and processed using a commercial kit (Oncogene Research Products, Boston, MA) according to the manufacturer's protocol. Sections were analyzed using the Eclipse E600 microscope (Nikon, Tokyo, Japan). Apoptotic cells (dark spots) were counted in 10 random high-power fields.

LG Organ Culture Ex Vivo

LG organ culture was performed as previously described.[16] Briefly, LGs were isolated and washed three times with sterile RPMI solution. Afterward, they were placed on the upper chamber of a Transwell system (1.2 μm-pore sized, Corning, Inc., Canton, NY) and immersed in an organ culture medium with or without 1.0 or 10 μM anti-TRAIL neutralizing antibody (Abcam Inc.; ND50, 0.2-2.0 μM), control IgG (1.0 μM, Biolegend, Inc., San Diego, CA), control glutathione-S-transferase (GST; 50 ng/mL), soluble recombinant TRAIL (20 ng/mL and 50 ng/mL). Approximately 1.75 × 10^5 peripheral blood mononuclear cells (PBMCs) were placed in the

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lower chamber. To make single-cell suspensions of PBMCs, whole blood cells were separated by Histopaque 1083 (Sigma-Aldrich) density gradient and centrifuged as previously described.[17,18] At 2, 6, and 24 hours, culture supernatants were harvested for ELISA analysis and leukocytes were used for flow analysis to determine apoptosis and proliferation as described in the following section.

ELISA

The concentration of IL-1β and TNF-α (R&D Inc.) and IL-17A (BioLegend Inc.) in lysates of LGs were assessed using ELISA according to the manufacturer's protocols. Levels of TRAIL in culture supernatants were evaluated using ELISA (Abcam, Inc.). All the experiments were repeated three times with at least triplicated samples.

Flow Cytometry

Single-cell suspensions of four LGs from each condition were prepared by treating minced tissue fragments with 100 U/mL collagenase D and 15 µg/mL DNase (Sigma-Aldrich) for 40 minutes at 37°C. After blocking with 1 µg of unlabeled anti-FcrR antibody (clone 2.4G2), cells (1×10^6) were washed with RPMI 1640 and surface-stained with fluorochrome-conjugated anti-CD11b, anti-CD45, anti-TRAIL, anti-Annexin V (eBioscience, San Diego, CA), or the appropriate isotype controls and then analyzed with FACS (FACSCalibur, Becton-Dickinson, Mountain View, CA). For purification of $CD45^-$ acinar cells and $CD45^+$ leukocytes, single-cell suspensions of LGs were stained with fluorochrome-conjugated anti-CD45 and sorted using FACS ARIA (Becton-Dickinson).

Fluorescein Staining

Corneal erosion grading used in this study was performed as previously described.[19,20] Briefly, before observation, the mice were anesthetized by Zoletil (tiletamine HCl and zolazepam HCl, Virbac). Then, corneal epitheliopathy was evaluated by placing 2 µL of 2.5% sodium fluorescein (vital staining) on the ocular surface for 3 minutes and examined under cobalt blue light using a slit-lamp biomicroscope. To avoid interobserver variation, the fluorescein-stained area (an indicator of corneal defects) were observed by two independent observers (Y.W.J. and E.Y.C.) and the calculated mean value from the erosion scores were used. Punctate staining was evaluated using the Oxford Scheme grading system and a grade of 0 to 4 were assigned.

Statistical Analysis

Continuous variables were examined for normal distribution by the Kolmogorov-Smirnov test. Variables that deviated from normal distribution were presented in mean and standard deviation, and those that were not normally distributed were presented as the median and interquartile range. For two-sample analysis, Mann-Whitney *U* test or Student's *t*-test were used. Kruskal-Wallis or one-way ANOVA was used to make comparisons among three or more groups. Bonferroni-corrected Dunnett's procedure was used as a post hoc analysis to compare each treated group to the control group. Statistical analysis was performed using SAS (version 9.13; SAS, Cary, NC) and R (version 3.2.5; Statistics and Mathematics, Vienna, Austria). Results are presented as the mean $\pm$ standard deviation. *P* value < 0.05 was considered significant.

Results

HIF-1α Regulates LG Inflammation and Inhibits Acinar Cells Apoptosis

HIF-1α is a transcriptional regulator that promotes cell survival during hypoxia-related pathological conditions.[21,22] To investigate whether HIF-1α regulates LG inflammation following desiccating stress, we utilized a widely used murine model of DED.[15,23,24] As a result, the qPCR experiment demonstrated a significant 3- to 4-fold increase in expression of HIF-1α transcript in the LGs of DED mice, compared with naive controls (Fig. 1A). Consistent with the mRNA analysis, our immunoblot data further confirmed the higher expression of HIF-1α at protein levels in DED-LGs (Fig. 1B). Next, IHC staining was performed to determine the cellular expression of HIF-1α in the LG. HIF-1α expression was observed primarily in acinar cells (indicated by the yellow arrowheads), with undetectable levels in ductal cells and infiltrating leukocytes (Fig. 1C). Using genetically modified mice in which HIF-1α was conditionally deleted in LGs (HIF-1α CKO mice),[15] we assessed whether HIF-1α deficiency augments inflammatory responses in LG during DED. Single-cell suspension of harvested LGs was prepared on day 7 and day 10, and flow cytometry analysis was performed. HIF-1α deficiency significantly increased the infiltration of $CD45^+$ inflammatory cells in the LG following DED induction, compared with the wild-type (WT) controls (Fig. 1D). Furthermore, following DED induction, increased frequencies of annexin V^+ $CD45^-$ acinar cells were observed in the LGs of HIF-1α CKO mice compared with the WT controls, suggesting that HIF-1α promotes the survival of acinar cells (Fig. 1D). Additionally, DED inductions resulted in significantly higher levels of inflammatory cytokines, IL-1β, IL-17A, and TNF-α in the LGs of HIF-1α CKO mice, relative to the WT control (Figs. 1E-G).

TRAIL Expression Increases in LG During DED

HIF-1α has been shown to induce the expression of death ligand PDL-1 in tumor cells.[25] Given the increased levels HIF-1α following DED induction, LG of DED mice were screened for the expression of potential death ligands that have been shown to regulate the immune response in various disorders.[26–28] The expression of TRAIL, PDL-1, and FAS-L was evaluated in the LGs on day 7 following DED induction (Fig. 2A). Desiccating stress significantly increased (3-fold) mRNA expression of TRAIL and did not alter the expression of PDL-1. Additionally, low levels of FAS-L were observed in DED-LG. Consistent with the mRNA analysis, our immunoblot data demonstrated increased protein level of TRAIL after DED induction (Fig. 2B). Unlike the LG, the cornea, brain, and skin did not express high levels of TRAIL in DED mice (Fig. 2C).

Previous studies have reported TRAIL expression in both immune and nonimmune cells, including keratinocytes,[29,30] fibroblasts,[31] and glandular cells.[32] We identified the cellular source of TRAIL in the DED-LGs using flow cytometry and IHC staining. Flow cytometry analysis demonstrated that the expression of TRAIL was significantly increased in the $CD45^-$ acinar cells yet was absent in $CD45^+$ immune cells after DED induction (Fig. 2D). Expression of TRAIL

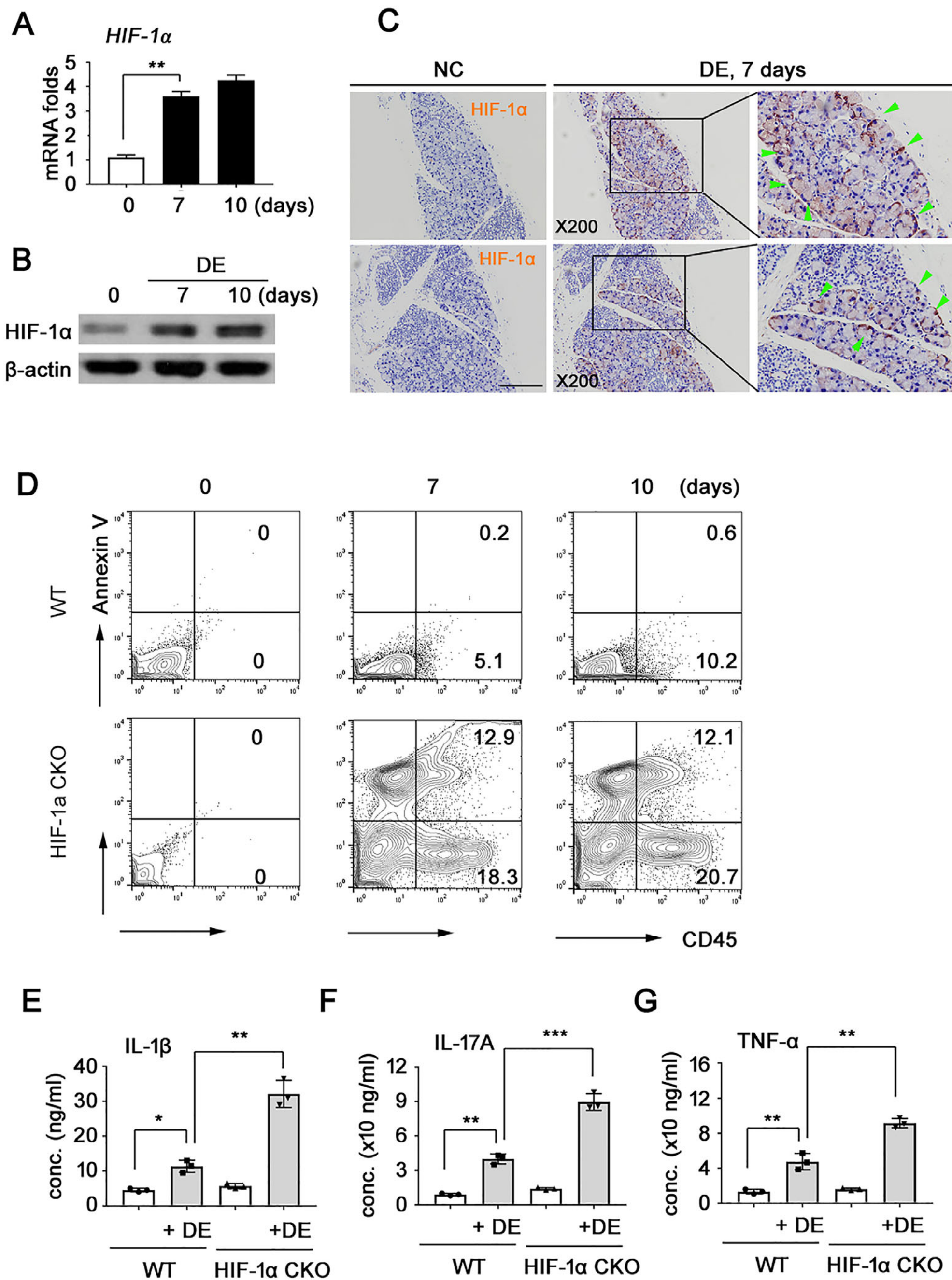

Figure 1. HIF-1α suppresses apoptosis of acinar cells and regulates lacrimal gland inflammation. (**A**) Real-time qPCR and (**B**) immunoblot analysis of HIF-1α in DED induced LGs (n = 5/group; **P < 0.01; one-way ANOVA, Kruskal-Wallis post hoc analysis). (**C**) Representative image of immunohistochemical staining of HIF-1α in LG on indicated days following DED induction. Green arrowheads indicate staining of HIF-1α in acinar cells (scale bar = 200 µm). (**D**) Representative flow cytometric plots showing increased infiltration of CD45$^+$ immune cells and apoptosis of CD45$^-$ acinar cells in LGs harvested from WT and HIF-1α CKO mouse on indicated days after DED induction (n = 6/group). ELISA analysis of (**E**) IL-1β, (**F**) IL-17A, and (**G**) TNF-α concentrations in lysates of LGs of WT and HIF-1α CKO mouse harvested on 7 day post-DED induction (n = 6/group; *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA, Kruskal-Wallis post hoc analysis). The data were from three independent experiments.

was further visualized in the acinar cell using IHC staining (Fig. 2E).

The function of TRAIL is largely determined by the receptor dominantly expressed on the target cells.[33] To investigate the immunoregulatory effect of TRAIL in DED, we evaluated the expression of DR5 (TRAIL receptor) and mDcR1 (decoy receptor) on FACS-sorted immune cells, and acinar cells of the LGs harvested from DED mice. As demonstrated by immunoblot analysis, CD45$^+$ inflammatory cells exhibited higher levels of DR5 and lower levels of mDcR1, compared to CD45$^-$ acinar cells (Fig. 2F). Our results collectively indicate that the TRAIL-DR5 axis regulates the infiltration of CD45$^+$ immune cells during DED.

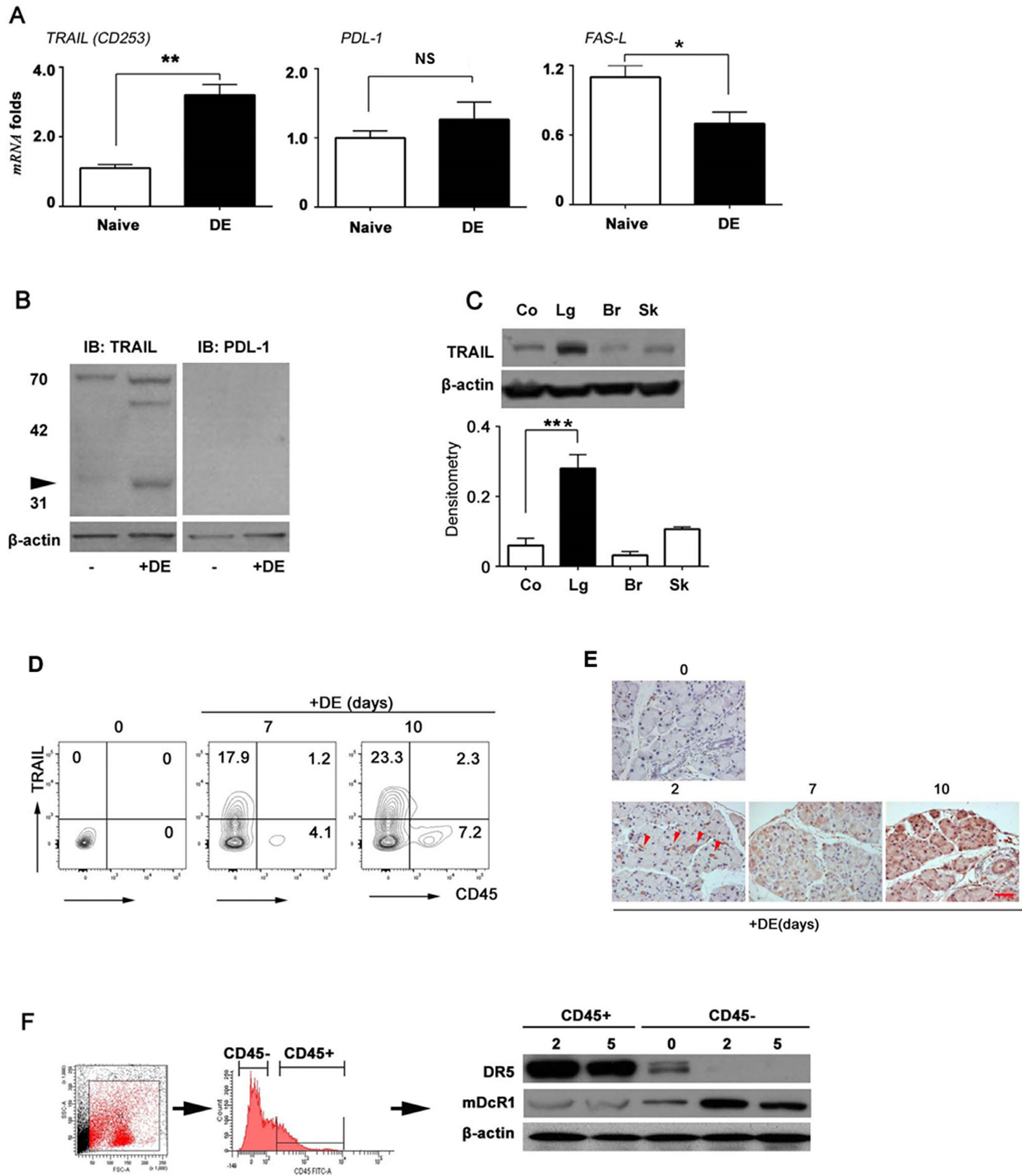

Figure 2. TRAIL expression increases in lacrimal gland during dry eye disease. (**A**) Real-time qPCR analysis of TRAIL, PDL-1, and FAS-L mRNA levels in LGs harvested on day 7 post-DED induction (n = 6/group; *$P < 0.05$, **$P < 0.01$, NS: not significant; one-way ANOVA, Kruskal-Wallis posthoc analysis). (**B**) Representative immunoblot images showing protein expression of TRAIL, PDL-1 in LGs harvested on day 7 post-DED induction. All experiments were repeated at least three times in triplicate. (**C**) Representative immunoblot image and cumulative bar chart of TRAIL expression on day 7 of DED induction in the cornea (Co), LG, brain (Br), and skin (Sk) (n = 6/group; ***$P < 0.001$; one-way ANOVA, Kruskal-Wallis post hoc analysis). (**D**) Representative flow cytometric plots showing $CD45^{-}TRAIL^{+}$ acinar cells and $CD45^{+}$ $TRAIL^{-}$ leukocytes in LG on indicated days following DED induction (n = 6/group). (**E**) Immunohistochemical staining of LG showing TRAIL expression on indicated days post-DED induction. Red arrowheads indicate TRAIL positive acinar cells. Representative images were selected from three independent experiments (scale bar = 50 µm). (**F**) Flow cytometry dot plot and histogram showing gating strategy for sorting $CD45^{-}$ acinar cells and $CD45^{+}$ leukocytes from single-cell suspension of LGs (left). Representative immunoblot showing DR5 and mDcR1expression on sorted $CD45^{-}$ acinar cells and $CD45^{+}$ leukocytes on indicated days post-DED induction (n = 6/group). The data were from three independent experiments.

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HIF-1α Promotes Apoptosis of Immune Cells by Inducing TRAIL Expression in the LG During DED

Our observation of increased expression of both HIF-1α and TRAIL in DED mice led us to investigate whether HIF-1α regulates TRAIL expression in the LG during DED. Desiccating stress was induced in WT, and HIF-1α CKO and LGs were harvested on day 7. DED induction resulted in approximately 2.5-fold increase in TRAIL expression at the mRNA and protein level, yet this effect was abrogated in HIF-1α-deficient mice (Figs. 3A, 3B). Our findings suggest that DED-induced TRAIL expression is dependent on acinar cell-derived HIF-1α.

Using our ex vivo LG-immune cell coculture system, we investigated whether LG induces apoptosis of immune cells via HIF-1α-mediated TRAIL secretion following DED (Fig. 3C).[16] Consistent with our in vivo observation, the ELISA analysis demonstrated a significant increase in secretion of TRAIL in culture supernatants of DED-LGs, compared with naive control cultures; yet, this increase was not observed in the supernatants of DED induced HIF-1α-CKO-LG (Figs. 3D, 3E). Moreover, DED-induced LG significantly amplified the frequencies of annexin-V$^+$ apoptotic leukocyte at 2, 6, and 24 hours after coculture, relative to naive control (Fig. 3F). The coculture was treated with TRAIL-neutralizing antibodies to confirm whether TRAIL promotes apoptosis of immune cells. Neutralization of soluble TRAIL reduced the frequency of apoptotic immune cells in a dose-dependent manner in DED-LG cultures, compared to the IgG-treated control (Fig. 3G). Using a gain of function approach, we determined whether HIF-1α promotes apoptosis of inflammatory cell in a TRAIL-dependent manner. Cocultures of the LGs of HIF-1α CKO and immune cells were performed in the presence and absence of recombinant TRAIL. Our data further demonstrated that DED-LGs of HIF-1α CKO mice did not induce the apoptosis of immune cells and addition of recombinant TRAIL resulted in increased frequencies of annexin V$^+$ immune cells, compared with GST-treated control (Fig. 3H). Our data collectively demonstrate that HIF-1α promotes apoptosis of immune cells by inducing TRAIL expression in LGs in DED.

HIF1α and TRAIL Insufficiency Results in Enhanced LG Inflammation and Epitheliopathy in DED

Given that knockdown of HIF-1α abrogates the expression of TRAIL following DED induction (Fig. 3A), we used HIF-1α CKO mice to investigate whether insufficiency of both HIF-1α and TRAIL lead to enhanced recruitment of inflammatory cells to the DED-LG and subsequently exacerbate ocular surface damage. Infiltrating immune cells were quantified using flow cytometry analysis. HIF-1α CKO DE-LG exhibited a significant increase in infiltration of CD45$^+$ total leukocyte (3.7-fold) and CD11b$^+$ cells (5.7-fold), compared with WT controls (Fig. 4A). IHC data visualized the increased frequencies of CD11b$^+$ immune cells in the LG of HIF-1α CKO on day 7 of DED induction (Fig. 4B). Furthermore, TUNEL analysis showed increased apoptosis of acinar cells (blue dots) in interlobular space of the LG from HIF-1α CKO mice after DED induction, relative to WT control (Fig. 4C).

Finally, to confirm the effect of HIF-1α and TRAIL on the pathogenesis of DED, fluorescein staining of ocular surface was performed in both WT and HIF-1α KO mice following DED induction (Fig. 4D). HIF-1α CKO mice displayed higher corneal erosion scores compared to the WT control, as indicated by a larger fluorescein stained area in the cornea.

Discussion

The present study provides novel insight into the role of HIF-1α-mediated TRAIL expression in regulating LG inflammation in DED. Specifically, our data show that (1) HIF-1α is critical for TRAIL expression, (2) acinar cells express elevated levels of TRAIL following desiccating stress, (3) TRAIL and its receptor system selectively promote the apoptosis of infiltrating leukocytes, and (4) HIF-1α and TRAIL insufficiency result in enhanced LG inflammation and advanced epitheliopathy in DED. Taken together, the signal cascade from HIF-1α mediated TRAIL expression may be a critical pathway of the innate protective mechanisms of LGs from desiccating stress-induced inflammatory cell infiltration.

Though it is an old concept, the "lacrimal functional unit" is still an actively working hypothesis describing DED as a disturbance of the integrated system comprising of the LGs, corneoconjunctiva, and neural networks connecting them all to each other.[1] Thus, if any component of this unit is compromised, DED may occur. Within this functional unit, because it is the main pathologic location and easily accessible, the majority of clinicians and researchers have been focused on the corneoconjunctival area to investigate inflammatory markers: measuring the treatment effectiveness of various drugs and determining the immunoinflammatory mechanisms.[15,19] In contrast to the ocular surface, however, it is true that the LG has been investigated mostly as an "organ" for tear production and less in terms of its immunoinflammatory mechanisms.

It is well known that extensive activation and infiltration of pathogenic immune cells including monocytes and macrophages, result in the impaired protective function of the LGs, leading to acute DED.[2,23] In our study, inflammatory cell infiltration and elevated TRAIL expression were observed beginning from 48 hours after DED induction (data not shown). And the TRAIL expression in acinar cells appeared to regulate inflammatory damage by inducing apoptosis of the infiltrating leukocytes during DED. Similar to the ocular surface, our data strongly support the idea that immunoinflammatory mechanisms in the LGs are important and are activated in the LGs from an early phase of DED. In accordance with these data, it is true that the number of immunoinflammatory molecule expressions are three times higher in the LGs compared with the ocular surface.[34]

HIF-1α has been reported to promote cell survival of immune and nonimmune cells during hypoxia.[35] Our previous report has shown that HIF-1α mediated autophagy signals promote acinar cell survival during desiccating stress.[15] An in vitro study in which HIF-1α was knocked down in myeloid cell has demonstrated a direct involvement of HIF-1α in regulating neutrophil survival in hypoxia.[36] We observed that HIF-1α prevents apoptosis of acinar cell in DED, corroborating our previous report.[35]

As a further study from our previous report showing elevated HIF-1α expression in DED-induced acinar cells, we hereby report elevated secretion of TRAIL in the LGs during DED through the activation of HIF-1α. TRAIL has been shown to induce apoptosis of various tumor cells and has been implicated as a potential therapeutic target

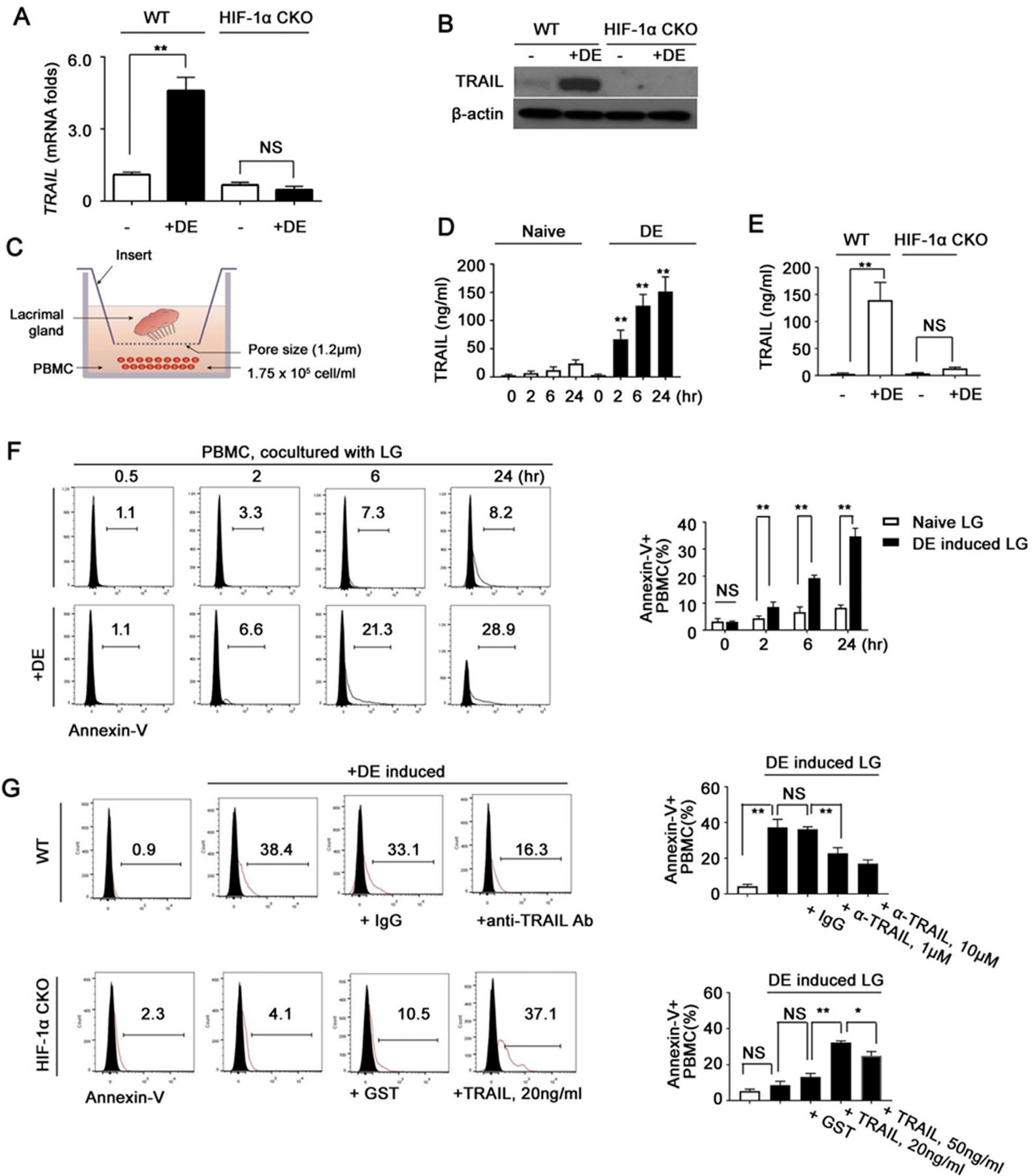

Figure 3. HIF-1α promotes apoptosis of immune cells by inducing TRAIL expression in the lacrimal gland during DED. (**A**) Real-time PCR analysis of TRAIL mRNA levels in WT and HIF-1α conditional knockout (CKO) mice at baseline and day 7 of DED induction (n = 6/group; $^{**}P < 0.01$). (**B**) Representative immunoblot image of TRAIL protein expression in WT and HIF-1α CKO mice at baseline and day 7 of DED induction. (**C**) Schematic diagram of ex vivo coculture of LG and PBMC. (**D**) ELISA analysis evaluating soluble TRAIL expression in the supernatant of naïve and DED-induced LG cocultured with PBMC for indicated time points. All experiments were repeated three times in triplicate (n = 6/group; $^{**}P < 0.01$ from the baseline; one-way ANOVA). (**E**) ELISA analysis evaluating soluble TRAIL expression in supernatant collected from the cocultures of WT or HIF-1α CKO LG (with or without DED induction) and PBMC at 24 hours. All experiments were repeated three times in triplicate (n = 6/group; $^{**}P < 0.01$ from the baseline; one-way ANOVA). (**F**) Representative flow cytometry data (left) and cumulative bar chart (right) showing annexin-V^+ PBMC cocultured with naïve or DED-induced LGs for indicated time points (n = 6/group; $^{**}P < 0.01$; one-way ANOVA). (**G**) Representative flow cytometry data (left) and cumulative bar chart (right) showing annexin-V^+ PBMC cocultured with LGs (harvested from naive or DED-induced WT mice) in the presence of control IgG (1.0 μM) and anti-TRAIL antibody (1.0 μM, and 10 μM) for 24 hours (n = 6/group; $^{**}P < 0.01$; one-way ANOVA) . (**H**) Representative flow cytometry data (left) and cumulative bar chart (right) showing annexin-V^+ PBMC cocultured with LGs (harvested from naive or DED-induced HIF-1α CKO mice) in the presence of control GST (50 ng/mL) or recombinant TRAIL protein (20 ng/mL and 50 ng/mL) for 24 hours (n = 6/group; $^{*}P < 0.05$, $^{**}P < 0.01$; one-way ANOVA, NS: not significant). All the experiments were repeated at least three times.

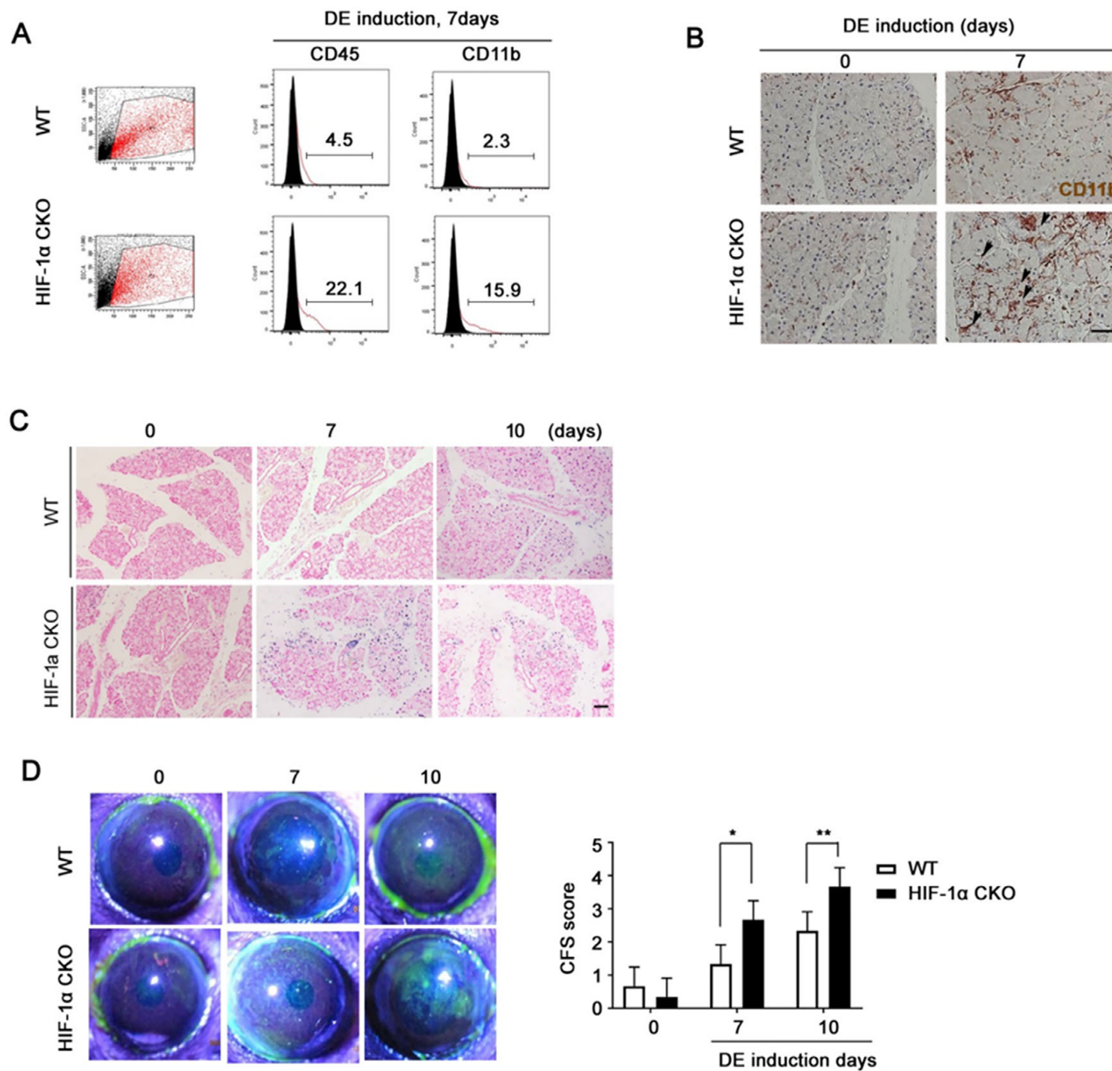

Figure 4. HIF-1α and TRAIL insufficiency results in enhanced lacrimal gland inflammation and epitheliopathy in DED. (**A**) Representative flow cytometry dot plots and histograms showing the infiltration of total CD45$^+$ leukocytes and CD11b$^+$ cells in DED-induced LG from WT and HIF-1α CKO mice on day 7 of DED induction (n = 6/group). (**B**) Representative immunohistochemical image of CD11b$^+$ cells in naïve and DED-induced LGs of WT and HIF-1α CKO mice on indicated days following DED induction (scale bar = 50 μm). (**C**) Representative microscopic images showing TUNEL$^+$ apoptotic cells (blue dots) in cross-sections of DED-induced LG from WT and HIF-1α CKO mouse on indicated days (scale bar = 50 μm). (**D**) Representative corneal fluorescein staining images (left) and clinical scores of corneal erosions (right) of WT and HIF-1α CKO mice on indicated days following DED induction. Experiments were repeated at least three times (n = 5/group; *$P < 0.05$, **$P < 0.01$; Student's *t*-test).

for cancer.[37,38] Our ex vivo coculture experiment reveals that TRAIL induces apoptosis of leukocytes during desiccating stress as evidenced by reduced frequency of annexin V$^+$ apoptotic immune cell in DED-LGs following neutralization of soluble TRAIL. Furthermore, our gain-of-function approach demonstrates that supplementation of recombinant TRAIL in TRAIL-deficient cultures of HIF-1α CKO LG significantly promotes immune cell apoptosis following DED.

TRAIL-induced apoptosis is regulated by TRAIL receptors expressed on target cells.[33] Decoy receptors inhibit activation of the apoptotic signaling cascade by either sequestering TRAIL from the receptors or by forming inactive heteromeric DcR1-DR5 complexes.[39,40] High expression of decoy receptors on tumor cells such as acute myeloid leukemia has been linked with poor prognosis.[29,41] Moreover, the modulatory function of the TRAIL-receptor system has been reported in various autoimmune disorders.[42] As demonstrated by our data, acinar cells exhibit higher expression of mDcR1, whereas infiltrating leukocytes express DR5 during DED induction. This dichotomy explains the selective apoptosis of CD45$^+$ cells but not acinar cells in the LG following DED induction, despite the elevated levels of TRAIL observed. Our study suggests that acinar cells use the decoy receptor system as a defense mechanism against TRAIL-induced apoptosis in DED.

In addition to receptor-regulation, the degree of TRAIL function is determined by the level of TRAIL expressed in the inflammatory environment. Previous studies have found TRAIL to be expressed in infiltrating mononuclear cells[43] and NK cells[44] in autoimmune disorders. Given the previous findings and the concurrent increase in leukocyte infiltration

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and TRAIL expression during DED, we hypothesized that the infiltrating leukocytes are the primary source of TRAIL. Interestingly, our study shows that TRAIL in DED is expressed by acinar cells of the LG, not the infiltrating leukocytes.

Our study illustrates that HIF-1α regulates TRAIL expression in DED as revealed by a deficiency of TRAIL expression in HIF-1α conditional knockout mice. Moreover, deficiency of HIF-1α results in elevated immune cell infiltration following DED induction. HIF-1α has been associated with immune-mediated disorders, including rheumatoid arthritis[45] and tumors.[46] The present study further delineates the immunoregulatory mechanism of HIF-1α in LG inflammation by demonstrating HIF-1α mediates TRAIL expression in DED. Furthermore, deficiency of both HIF-1α and TRAIL not only promote the infiltration of immune cell in LGs but also results in increased levels of inflammatory cytokines. Therefore, corneal staining results in DED may reflect the global effect of altered HIF-1α and TRAIL status on the lacrimal functional unit because it has an impact on the ocular surface epithelium.

There are several limitations to this study. First, aside from HIF-1α activation, an upstream signal for TRAIL induction was not identified. However, it is still unclear whether the damage-associated molecular pattern signal or other neural or hormonal factors may be the main activator of TRAIL expression in acinar cells. The second limitation is that we used male B6 mice for this study. The expression levels of TRAIL might be differentially affected by sex, aging, and other types of LGs (extra- or intraocular). Additionally, it is possible that the human TRAIL system is different from that of mice. Therefore, the present findings regarding TRAIL expression should only be applied and interpreted to the murine DED model.

In conclusion, this study highlights the immunoregulatory function of TRAIL and its receptor system on the LG in DED. TRAIL secretion is regulated by acinar cell-derived HIF-1α and prevents inflammatory damage by promoting apoptosis of infiltrating leukocytes. Moreover, differential upregulations of DR5 on leukocytes and DcR on acinar cells induce selective apoptosis of infiltrating leukocytes in the LG. These observations could be implicated in designing a potential TRAIL-based therapeutic strategy to modulate LG inflammation in DED.

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