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Non-immune and immune functions of Interleukin-36γ suppress epithelial repair at the ocular surface

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Abstract

Regulation of innate inflammation is critical for maintaining tissue homeostasis and barrier function, especially in those interfacing the external environment such as the skin and cornea. Expression of pro-inflammatory cytokines by injured tissues has been shown to exacerbate the inflammatory cascade, causing tissue damage. Interleukin 36 cytokine, a subfamily of the IL-1 superfamily, consists of three pro-inflammatory agonists IL36α, IL36β, and IL36γ and an IL36 receptor antagonist (IL36Ra). The current investigation, for the first time, reports that IL36γ is the primary isoform expressed by the corneal epithelium, which is significantly upregulated following corneal injury. The function of IL36γ on non-immune cells, in addition to innate inflammatory cells, in regulating tissue homeostasis has not been well investigated. Using a loss-of-function approach via neutralizing antibody treatment, our data demonstrate that blocking endogenously-expressed IL36γ in epithelial cells promotes rapid repithelialization in *in vitro* wound closure assay. Finally, by utilizing a naturally occurring antagonist IL36Ra in a well-established murine model of ocular injury, our study demonstrates that inhibition of IL36γ accelerates epithelial regeneration and suppress tissue inflammation. Given rapid wound healing is critical for re-establishing normal tissue structure and function, our investigation on the function of IL36γ provides evidence for the development of novel IL36γ-targeting strategies to promote tissue repair.

Keywords

Interleukin 36; Interleukin36γ blockade; Epithelium; Tissue injury; Tissue regeneration; Innate inflammation

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AUTHOR CONTRIBUTIONS

S.S. assisted in designing the study, performed the experiments and analyzed the data and wrote the manuscript. W.C. assisted in performing the experiments, data analysis and manuscript writing. E.E., R.B.S, assisted in performing the experiment. S.K.M. and S.K.C. contributed to the underlying hypothesis, designed the study, analyzed data, and wrote the manuscript.

CONFLICT OF INTEREST

The authors of this manuscript have no conflicts of interest to disclose.

Rapid wound healing allows for faster restoration of physiological functions of tissues such as corneal transparency for light transmission following ocular trauma, infections, and transplantation. Tissue injuries are hallmarked by epithelial damage and subsequent influx of immune cells and secretion of inflammatory mediators.[1] During tissue repair, injury-induced immune response accelerates the clearing of tissue debris, and epithelial cells from wound edges migrate to re-epithelize the lesion.[2] However, excessive activation of innate immune cells, including macrophages and neutrophils, and impaired re-epithelization cause severe chronic wounds.[3] The current treatment of choice to aid wound healing, such as corticosteroid, not only non-specifically suppresses the immune response but impedes the process of re-epithelization, resulting in an increased risk of infections and malignancies.[4] Given the strong correlation between delayed wound healing and the severity of inflammation, understanding the core mechanism that regulates both these phenomena is critical for the development of new therapeutic modalities.

Interleukin 36 (IL36), a group of cytokines, mediates inflammatory response by binding to the IL36 receptor (IL1RL2). The three isoforms IL36α, IL36β, and IL36γ have been shown to be involved in the autoimmune pathogenesis of skin, lungs, and intestine.[5] Elevated levels of IL36α and IL36γ in the serum of patients with systemic lupus erythematosus have been reported.[6] High expression of IL36γ, at mRNA and protein levels, was observed in the skin of psoriasis patients.[7] Recently, IL36α and IL36β have been reported to activate cells of adaptive immunity, including dendritic cells and CD4+T cells, in a murine model of skin inflammations.[8] Despite the amalgam of previous reports on the importance of IL36 cytokines in inflammatory disorders, no study has investigated whether IL36 cytokines contribute to epithelial regeneration and tissue inflammation following corneal injury.

The purpose of our study was to analyze the function of IL-36 cytokines in modulating epithelial repair and corneal inflammation following injury. In this study, we utilized a widely used and well-standardized murine model of corneal injury that allows the real-time assessment of epithelial regeneration.[9,10] The paucity of immune cells in a naive state makes the cornea an excellent in vivo system to investigate the infiltration of immune cells.

Here, we evaluated the expression of IL-36 isoforms (IL-36α, -36β, -36γ) in naïve and injured corneas and showed that IL36γ is primarily produced by corneal epithelium and upregulated following corneal injury. In addition, we utilized the gain- and loss-of-function approach to determine the biological function of IL36γ at the ocular surface using in vitro and in vivo models. Our data demonstrate that epithelium-derived IL-36γ negatively impacts wound healing by suppressing epithelial regeneration and promoting ocular infiltration of activated macrophages and neutrophils following corneal injury.

METHODS

Mice

Six- to eight-week-old male and female C57BL/6 wild-type mice (Charles River Laboratories, Wilmington, MA) were used for *in vivo* experiments. The study protocol (2020N000186) was approved by the Schepens Eye Research Institute Animal Care and Use

Committee. All mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal injury

A hand-held motor brush (Algerbrush II, Alger Company Inc., Lago Vista, 75 TX, USA) was used to induce corneal injury in the right eye of mice under general anesthesia, as described previously. [9,10] Briefly, mice were anaesthetized, central cornea was marked with a 2mm trephine and corneal epithelium was removed mechanically using an Algerbrush. Triple antibiotic ointment (Neomycin and Polymyxin B Sulfates and Bacitracin Zinc Ophthalmic Ointment USP, Bausch + Lomb, Wilmington, MA) was applied after injury followed by the subcutaneous injection of buprenorphine to minimize the pain. Recombinant IL-36Ra (0.2mg/ml, Cat. #760804, Biolegend, USA) was administered topically thrice a day for three days post-corneal injury to block the function of endogenous IL36γ. PBS-treated mice were used as controls.

Corneal fluorescein staining and slit lamp biomicroscopy

Healing of the wounded corneal epithelium was assessed using corneal fluorescein staining (CFS) and images were captured through slit lamp biomicroscope, as described previously.[9,11] To perform CFS, 1μl of 1% sodium fluorescein was placed on the injured ocular surface and corneal epitheliopathy was evaluated under cobalt blue light using slit lamp biomicroscope after 3 minutes. The corneal epitheliopathy was evaluated immediately following injury (day 0), as wells as every day till day 3 post-injury. Images were analyzed and area of epithelial defect was measured through ImageJ.

Flow cytometry

To evaluate infiltration of immune cells in corneas, a single-cell suspension was prepared by digesting harvested corneas using Collagenase IV (4mg/ml, Sigma-Aldrich, St. Louis, MO) and DNase I (2mg/ml, Roche, 88 Basel, Switzerland) using a protocol described previously.[12,13] The cells were stained with fluorochrome-conjugated anti-CD45 (clone: 30-F11), anti-CD11b (M1/70), anti-Ly-6G (1A8) antibodies, and with the respective isotypes. All of the antibodies and isotypes were purchased from Biolegend. The stained cells were analyzed on LSR-II flow cytometer (BD Biosciences, USA). The data were analyzed using Summit™ software (Dako Colorado, Inc., Fort Collins, CO, USA). The gating strategies to evaluate specific immune populations in single cell suspensions of the cornea are detailed in Supplementary Figure 1.

Cell Culture

Human corneal epithelial (HCEC) cells were received as gift from Dr. Ilene K. Gipson's laboratory (Schepens Eye Research Institute, Harvard Medical School, Boston, USA). The cells were cultured in Keratinocyte SFM medium (GIBCO, ThermoFisher Scientific, USA) under standard culture conditions (37°C and 5%CO_2). The medium was supplemented with bovine pituitary extract and epidermal growth factor (EGF, 0.2ng/ml, Gibco, USA), as per manufacturer's instructions. The medium was replaced every two days and cells were sub-cultured at 75% confluency. To assess the expression of IL36 receptor and

secretion of IL36γ by HCECs following injury, HCECs were stimulated with recombinant human IL-1β (100 ng/ml; Cat. #579402, Biolegend) for 24 hrs to mimic the inflammatory microenvironment following corneal injury. To generate human macrophages, THP1 human monocytic cell line was treated with 100nM 12-myristate 13-acetate (PMA; Cat. #P8139, Sigma-Aldrich, USA) for 72 hrs in RPMI-1640 medium supplemented with fetal bovine serum (Gibco, ThermoFisher Scientific, USA). Macrophages were stimulated with recombinant IL-36γ (100ng/ml; Cat. #767402, Biolegend®) for 24 hrs.

In vitro Scratch Assay

Human corneal epithelial cells (HCEC) were seeded in a 12-well plate and allowed to grow in Keratinocyte SFM medium until complete confluency and scratch assays were performed as described previously.[14] Briefly, a scratch was created in the center of the monolayer using a pipette tip and cells were incubated under standard culture conditions. Human IL-36γ/IL-1F9 neutralizing antibody (100ng/ml) (Cat. #AF2320, R&D Biosystems, USA) was added and cells were followed for 72 hours for healing of the scratched/wounded area. Images were taken under phase contrast microscope at regular intervals and analyzed using ImageJ software.

RNA isolation and real-time PCR

RNA isolation was performed using the RNeasy® Micro Kit (Cat. #74004, QIAGEN, Denmark) as per the manufacturer's instructions. Isolated RNA was quantified using NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific, USA) followed by reverse transcription to synthesize cDNA using oligo(dT) primer and SuperScript III First-Strand Synthesis System (ThermoFisher Scientific, USA). Real time PCR was performed using TaqMan® Universal PCR Mastermix (Life Technologies, ThermoFisher Scientific, USA) and pre-formulated TaqMan® primers for *Il36α (*Mm00457645_m1*), Il36β (*Mm01337545_m1*), and Il36γ (*Mm00463327_m1*), HLA-DR* (Human leukocyte antigen-DR; Hs00219578_m1), *Il1β* (Human: Hs01555410_m1, & Murine: Mm00434228_m1) *Tnfα (*Human; Hs00174128_m1 & Murine: Mm00443258_m1), *Il-10* (Mm01288386_m1) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh;* Human: Hs99999905_m1, & Murine: Mm99999915_g1) in the Mastercycler® RealPlex^2 platform (Eppendorf, Germany). The results were normalized to *Gapdh* (internal control) and analyzed using comparative threshold cycle method.

Enzyme-linked immunosorbent assay (ELISA)

Levels of IL36γ in murine corneal epithelium and whole cornea were analyzed using in-house ELISA method. To separate the corneal epithelium, harvested corneas were incubated in EDTA (diluted 1:25 in PBS) at 37°C for 30 minutes and epithelium was peeled off under the microscope using blunt-end tweezers. Corneal and HCEC lysates were prepared by allowing corneal tissue (in 0.1% Triton-x-100) to undergo 3 freeze-thaw cycles at −180°C/37°C, followed by homogenization with motorized pestle and centrifugation at 10,000 rpm for 10 minutes. Lysates were diluted with coating buffer (50:50) and plated (100μl/well) in the wells of 96 well-ELISA plate, followed by overnight incubation at 4°C. The wells were washed with washing buffer (DuoSet® ELISA kit, R&D Systems, USA), blocked with 2%BSA (Bovine Serum Albumin, Sigma-Aldrich, USA) for 1 hour, incubated with IL1F9/

Il-36γ antibody (Cat. # LS-C487612, LSBio, USA; dilution 1:50) for 3 hours followed by incubation with HRP conjugated anti-rabbit IgG antibody (Cat. #406401, Biolegend, USA) for 1 hour under continuous rocking. The wells were washed with washing buffer, incubated with substrate solution (100μl/well) for 10 minutes in the dark to allow for color development, followed by the addition of stop solution (100μl/well; DuoSet® ELISA kit, R&D Systems, USA). The results were analyzed using SpectraMax® Plus 384 microplate reader (Molecular Devices, USA).

Histology and immunofluorescence

Formalin-fixed paraffin-embedded corneal cross-sections were stained with hematoxylin and eosin (H&E) and examined under the brightfield microscope as described previously.[9,15] Number of epithelial cell layers displaying continuum of epithelial cells with intact nuclei were quantified. For immunofluorescence analysis, sections were de-paraffinized, blocked with 2%BSA and anti-FcR antibodies (catalog #14-0161-86, eBioscience, ThermoFisher Scientific, USA), and immunostained with Mouse IL-1Rrp2/IL-1R6 antibody (Cat. #AF2354, R&D Systems, USA; 1:50) overnight at 4°C. Slides were washed with washing buffer (0.5% Triton-X-100 and 2% FBS in PBS; 4 washes,15 minutes each) and incubated with Donkey anti-goat IgG TRITC antibody (Cat. #A16004, ThermoFisher Scientific, USA; 1:250) for 1 hour. Slides were washed in washing buffer (4 washes of 15 minutes each) and mounted using VECTASHIELD® mounting medium (Cat. # H-1200, Vector Laboratories, USA). The stained immunofluorescence slides were examined under a fluorescence microscope (Nikon® Eclipse E800).

Statistical analysis

Statistical significance ($p<0.05$) was determined by Student's t-tests. Results are presented as the mean ± SD of three independent experiments. Quantification of images of corneal injury, CFS, scratch assay, as well as *in vivo* evaluations, was performed in a masked fashion. Sample sizes were estimated based on previous reports on corneal injury and inflammation.[11,16,17]

RESULTS

IL-36γ is predominantly secreted by corneal epithelium following injury

Given the reports of high levels of IL36 cytokines (IL-36α, IL-36β, and IL36γ) in various autoimmune disorders,[18] we investigated the expression of IL-36 isoforms in the cornea in a homeostatic and injury-induced inflammatory environment. Whole cornea, corneal epithelium, and stroma were harvested from naïve mice and lysed to evaluate the expression of IL36α, -36β, and -36γ (Figure 1A). As evaluated by real-time PCR analysis, naïve epithelial cells expressed significant levels of IL36γ, which was 47- and 4-fold higher compared to IL-36α and IL-36β, respectively. Tissue expression of IL36γ was 55% lower in the corneal stroma relative to the epithelium ($p<0.01$) (Figure 1A). To evaluate the expression of IL36 isoforms following corneal injury, the epithelium and anterior stroma were mechanically removed using a handheld motor brush, and corneas were harvested at 24 hrs post-injury and corneal epithelium and stroma were separated for real-time PCR and ELISA analysis. The injured epithelium showed a significant increase in expression of

IL36γ at mRNA ($p<0.001$) and protein levels ($p<0.05$) compared to that of naïve controls and injured stroma (Figures 1A & 1C). Moreover, IL36γ expression was significantly higher compared to the expression of IL-36α and IL-36β in the epithelium following injury. The expression of IL36γ was further evaluated in whole corneal lysates. Akin to the epithelium, the expression of IL36γ was significantly elevated in injured corneas compared to naïve controls, with a 10-fold increase at mRNA levels ($p<0.001$) and a 4-fold increase at protein levels ($p<0.05$) (Figures 1B and 1C). Furthermore, protein expression of IL36γ was confirmed in resting and inflamed human corneal epithelial cells (HCECs) as demonstrated by constitutively high secretion of IL36γ by both resting and IL1β-stimulated HCECs (Figure 1D). Our data indicate that epithelium constitutively expresses substantial levels of IL36γ and serves as the primary source of upregulated IL36γ at the ocular surface following corneal injury.

IL-36 receptor is expressed by epithelium and immune cells at the ocular surface

IL36γ has been reported to exert pro-inflammatory functions by binding to the lL-36 receptor (IL36R) expressed on target cells during autoimmune disorders.[19] To evaluate the expression of IL36R at the ocular surface, corneas were harvested from naïve mice at 24hr following injury. Cross-sections of harvested corneas were stained with anti-IL36R antibodies for immunohistochemistry analysis. The results demonstrated constitutive expression of IL36R by the epithelium of naïve and injured corneas (white arrowheads, Figure 2A). Given the previous reports and our observation of increased infiltration of immune cells following corneal injury,[15,20] we next evaluated the expression of IL36R on infiltrating CD45+ total immune cells, Ly6G+CD11b+ neutrophils, Ly6G-CD11b+ macrophages, and CD45− cells (primarily epithelial cells) at the ocular surface. At 24h post-injury, corneas were harvested and digested into a single-cell suspension to quantify IL36R expression using flow cytometry. Consistent with the immunohistochemistry data, histogram analysis showed a significant expression of IL36R on CD45− epithelial cells (Figure 2B). Moreover, CD45+ immune cells were observed to express a significantly high level of IL36R, as demonstrated by a 3-fold higher expression of IL36R compared to control isotype-stained cells (Figure 2B). In addition, further flow cytometry analysis of ocular surface immune cells revealed significant expression of IL36R on neutrophils (2-fold) and macrophages (6-fold) compared to isotype controls (Figure 2C). Consistent with the murine data, HCECs expressed substantial levels of IL36R following IL1β stimulation (Figure 2D).

IL-36γ delays wound closure of human corneal epithelium and promotes macrophage activation in vitro

Having observed upregulated secretion of IL36γ and constitutive expression of its receptor by both murine and human corneal epithelial cells, we next investigated whether endogenous IL36γ directly regulates epithelial regeneration. To determine this, we performed a scratch wound healing assay in which a wound was created by creating a uniform scratch in a monolayer of cultured primary human corneal epithelial cells (HCECs).[14] Healing of the scratch was monitored for 72 hours under a phase-contrast microscope in the presence of an IL-36γ neutralizing antibody (100ng/ml). The isotype-treated cultures served as controls. The area of the wound was calculated using ImageJ analysis of the brightfield images (Figure 3A). Blockade of IL-36γ significantly accelerated the rate of wound closure at

24 hours (1.57-fold; $p<0.05$), 48 hours (1.8 fold; $p<0.05$), and 72 hours (8-fold; $p<0.05$), relative to isotype-treated controls. Previous studies have shown that IL36γ promotes the activation of immune cells, including bone marrow dendritic cells and CD4+ spleen T cells.[21] Given our observation of high expression of IL36R by infiltrating macrophages at the ocular surface, we confirmed the effect of exogenous IL36γ on the activation of human macrophages. THP1-derived macrophages were stimulated with recombinant IL36γ (100ng/ml and 200ng/ ml) for 24h, and expression of activation marker HLA-DR, IL1β, and TNFα was evaluated using real-time PCR (Figures 3B–D). The addition of IL36γ resulted in a two-fold increase in the expression of HLA-DR ($p<0.01$) compared to untreated controls. Moreover, a significant increase in expression of IL1β ($p<0.001$) and TNFα ($p<0.05$) was observed following IL36γ stimulation, relative to the untreated control. Our results indicate that IL36γ directly exerts an inhibitory effect on the regeneration of human corneal epithelium and promotes the activation and pro-inflammatory function of macrophages.

Blockade of Il-36γ accelerates re-epithelialization following corneal injury

To evaluate the effect of blocking IL-36γ function on epithelial repair following corneal injury in vivo, recombinant IL-36 receptor antagonist (IL36Ra) was administered topically (0.2mg/ml) thrice a day for three days post-corneal injury (Figure 4A). Injured PBS-treated mice were used as controls. On day 3 post-injury, the efficacy of IL36Ra treatment was confirmed by evaluating the expression of IL36γ in harvested corneas. Real-time PCR analysis showed a 75% reduction in the expression of IL36γ following IL36Ra treatment compared to control PBS treatment ($p<0.001$; Figure 4B). Furthermore, corneal epitheliopathy was evaluated through corneal fluorescein staining (CFS) and images were analyzed using ImageJ software. IL36Ra treatment substantially accelerated the rate of epithelial repair following injury compared to PBS treatment (Figure 4C). Indeed, an 86% reduction in epithelial defect (green area) was observed by 24 hours post-injury in IL36Ra treated group, relative to the PBS-treated control ($p<0.05$; Figure 4C). To visualize normalization of corneal tissue architecture in the epithelium layer, corneas were harvested, and cross-sections were stained with H&E. IL36Ra treated corneas showed comparable corneal architecture to naïve corneas with significant restoration of the 4–5 epithelial cell layers, compared to the PBS-treated corneas ($p<0.05$; Figure 4D). Moreover, the epithelium was uniformly organized with smooth outer surface, compared to the PBS-treated controls showing disrupted and disorganized epithelium (Figure 4D). Unlike PBS-treated controls, IL36Ra treated corneas showed organized. Our data indicate that IL36γ delays wound healing by inhibiting faster re-epithelization and subsequent normalization of corneal tissue structure following injury.

Blockade of IL-36γ reduces infiltration of immune cells and expression of inflammatory cytokines following corneal injury

Previous reports showing the positive correlation between IL36γ expression and severity of tissue inflammations during skin and lung diseases,[22,23] led us to investigate whether IL36γ augments inflammatory response following corneal injury. IL36γ functions were attenuated by topical administration of recombinant IL36Ra (0.2mg/ml) thrice daily for three days post-corneal injury. Thereafter, corneas were harvested for real-time PCR

analysis of inflammatory and regulatory cytokines. Our data revealed more than 90% suppression in the expression of pro-inflammatory cytokines IL1β ($p<0.001$; Figure 5A) and TNFα ($p<0.001$; Figure 5B) following IL36Ra administration relative to PBS treatment. In contrast, expression of IL10, an anti-inflammatory cytokine,[24] was increased by 4-fold in injured corneas following IL-36Ra treatment, compared to PBS treatment ($p<0.05$; Figure 5C). Furthermore, to analyze the different subsets of immune cells involved in the wound healing cascade, corneas were harvested, and single-cell suspension was prepared to evaluate the infiltration of total $CD45^+CD11b^+Ly6G^+$ neutrophils and $CD45^+CD11b^+Ly6G^-$macrophages using flow cytometry (Figure 5D). Naïve and injured-PBS-treated mice were used as controls. IL36Ra treatment resulted in a significant 34% decline in total $CD45^+$ leukocyte infiltration, compared to PBS treatment ($p<0.05$; Figure 5E). Treatment with IL36Ra significantly reduced the frequencies of neutrophils ($p<0.01$; Figure 5F) and macrophages ($p<0.05$; Figure 5G), relative to PBS-treated controls. Our data suggest that IL36γ contributes to ocular inflammation by promoting infiltration of immune cells and the expression of pro-inflammatory cytokines following corneal injury.

DISCUSSION

This study highlights the function of epithelial-derived IL36γ in delaying epithelial regeneration and provoking ocular inflammation following corneal injury. Specifically, we show that corneal epithelium constitutively expresses IL36γ, a major isoform, which is upregulated following corneal injury. Antibody-mediated neutralization of endogenous IL36γ promotes faster wound closures in HCEC scratch assays, and the addition of recombinant IL36γ activates THP-derived macrophages. Finally, in vivo blockade of IL36γ accelerates epithelial wound repair, suppresses ocular inflammation, and normalizes tissue architecture following corneal injury.

Epithelium serves as the outermost protective barrier and maintains ocular immune homeostasis by expressing anti-inflammatory molecules at the cell surface. Previous studies have shown that disrupted epithelium secretes pro-inflammatory cytokines IL1β and TNFα to promote ocular inflammation in corneal disorders.[1] In models of bacterial keratitis, human epithelial cells have been shown to express varied isoforms of interleukin 36, a danger-associated molecule.[25,26] In detail, Gao et. al. have demonstrated increased expression of IL36α and IL36γ and exogenous administration of IL36Ra enhanced the severity of *Pseudomonas* infection, demonstrating the protective proinflammatory function of IL36 during bacterial infection. In another report, Me et. al. have shown that exogenous IL36α promote dendritic cell infiltration and Th2-mediated immune response to reduce the infectious burden of *Pseudomonas*. Beyond their protective pro-inflammatory function against bacterial load, our study for the first time highlights that corneal epithelium pre-stores constitutively high levels of IL36γ and exert its biological effect to retard wound healing primarily by directly suppressing epithelial regeneration.

In addition to secreting IL36γ, immune cells such as macrophages in various autoimmune disorders including psoriasis and Crohn's disease[27–29] have shown to express their cognate ligand IL36R. Consistent with the immune cells of the lymph node and skin,[19] ocular macrophages and neutrophils were also observed to express IL36R. In addition to

immune cells, our study further shows that corneal epithelial cells in naïve and injured corneas express IL36R, suggesting IL36γ exerts a direct effect on immune cells and epithelium following tissue injury. To evaluate the distinct effect of IL36γ on epithelial and immune cells, in vitro experiments utilizing cells of human origin were conducted. Our experiment neutralizing endogenous IL36γ levels in HCEC scratch assays demonstrates that IL36γ hinders epithelial regeneration. Furthermore, using a gain-of-function approach, we demonstrate that the addition of recombinant IL36γ promotes activation of human macrophages as shown by increased expression of inflammatory molecule IL1β and TNFα. Our in vitro experiments utilizing human cells provide strong proof-of-concept in human settings for translational implications.

Consistent with non-ocular tissue injuries of skin, lung and kidney,[30,31] we and others have reported that chemical and mechanical corneal injuries result in a rapid and enormous infiltration of neutrophil and macrophages to the ocular surface injury.[15,20] Given the substantial contribution of immune response to collateral tissue damage, novel strategies to prevent the recruitment and suppress the effector function of neutrophils and macrophages have attracted attention,[30,31] Neutrophil and macrophage exacerbate inflammation by secreting pro-inflammatory cytokine IL1β and TNFα.[11,16] In the current study, we show that epithelial-derived IL36γ promotes migration of immune cells to injured tissue, as indicated by a significant 34% reduction in the frequencies of total $CD45^+$ leukocytes in corneas treated with IL36RA. Previous studies have shown that corneal immune microenvironment is dependent not only on the frequencies but also specific immune cell subsets.[32,33] Our detailed analysis of immune cell subsets demonstrates IL36RA treatment results in approximately 47% and 24% reduction in the frequencies of neutrophils and macrophages, respectively, at the ocular surface. Moreover, we show that IL36γ promotes the activation of immune cells at the ocular surfaces as evidenced by the significant decrease in the expression of IL1β and TNFα following *in vivo* administration of IL36Ra in mice corneas. The pro-inflammatory function of IL36γ at the ocular surface was further corroborated by the increased expression of anti-inflammatory cytokine IL-10 in IL36Ra treated corneas.

Excessive tissue inflammation has been associated with delayed re-epithelization, which in severe conditions leads to ulceration and perforation.[9,11,34] The clinical evaluation using fluorescein-stained corneas demonstrates that topical blockade of IL36γ accelerates epithelial wound repair compared to PBS treatment. Indeed our in vivo data, in conjunction with the in vitro data of faster human epithelial cell regeneration in the scratch assays, suggest a novel inhibitory function IL36γ in epithelium regeneration. Although clinical evaluation allows real-time follow up of the epithelial wound repair, optimal restoration of corneal function necessitates the complete restoration of epithelial layers. Our H&E data evaluating the re-epithelization at the molecular level clearly demonstrate normalized stratification of epithelial layers as indicated by the uniformly organized five to six layers of epithelial cells following IL36Ra treatment. Of note, control PBS-treated corneas showed disrupted and disorganized epithelial layers despite the reduction in the corneal fluorescein staining.

In conclusion, the study provides evidence on the cellular source and pathogenic function of IL36γ at the ocular surface following corneal injury. Our data shows that corneal epithelium

serves as the major source of IL36γ, which impairs wound healing by suppressing epithelial regeneration and promoting ocular inflammation. Our experiment on the topical blockade of IL36γ provides a novel framework to develop targeted therapeutics for persistent epitheliopathy and inflammatory conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

Included in article: The data that support the findings of this study are available in the methods of this article. Detailed data that support the findings of this study are available from the corresponding author upon reasonable request.

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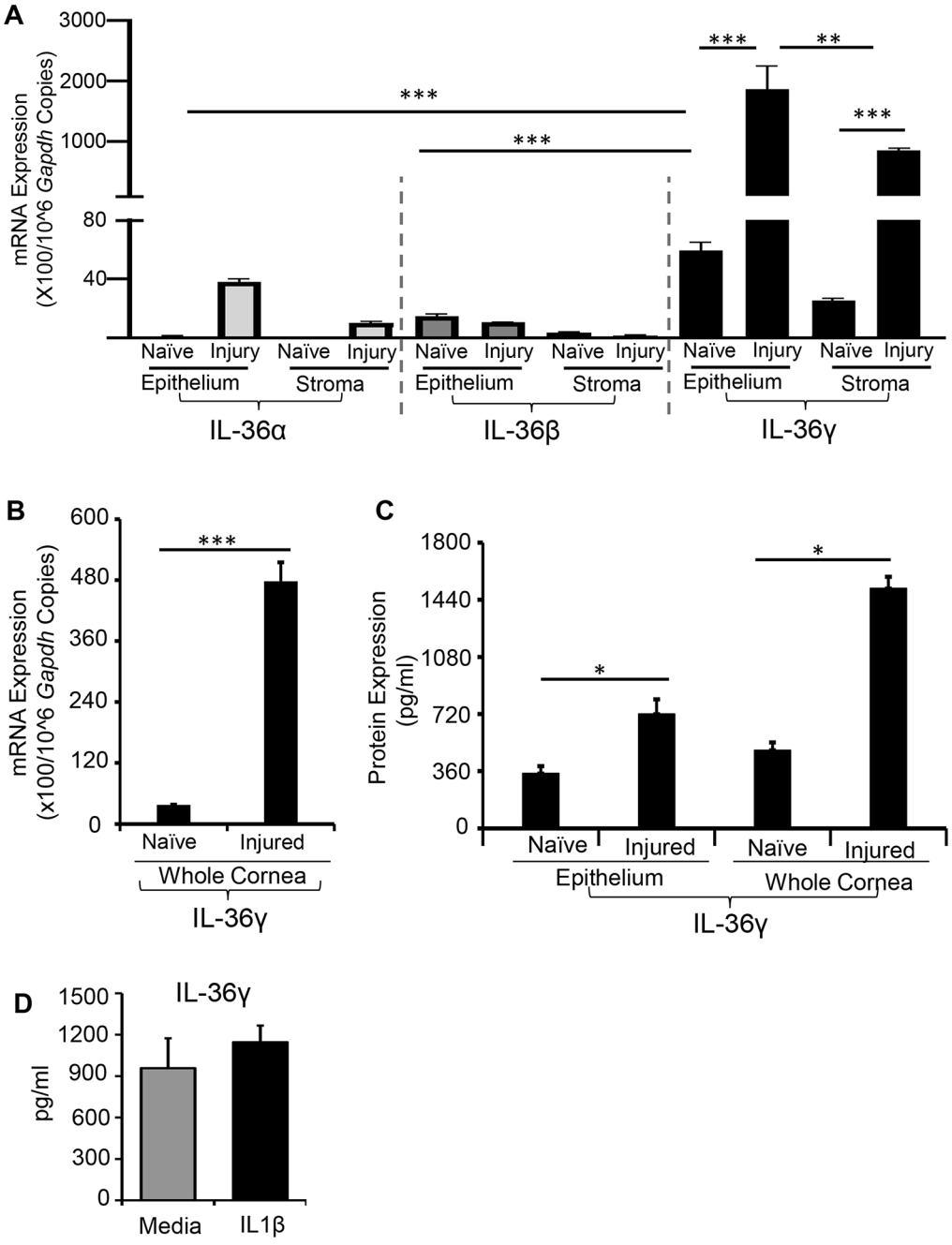

Figure 1. IL36γ is predominantly expressed by corneal epithelium and is significantly up regulated post-corneal injury.

Corneal injury was induced by the mechanical removal of the corneal epithelium and anterior stroma in C57BL/6 mice. At 24 hrs. post-injury, corneas were harvested to separate the epithelium and stromal tissues. Corneal tissue harvested from naïve mice served as control. **(A)** Real-time PCR analysis quantifying mRNA expression of IL-36 gene family (IL36-α, -β, -γ) in epithelium and stroma from injured and naïve corneas **(B)** Whole corneas were harvested from naïve and injured mice and IL-36γ mRNA levels were quantified using real-time PCR. **(C)** ELISA analysis quantifying protein levels of IL-36γ in lysates of corneal

epithelium and whole corneas collected from naïve and injured mice. (**D)** Human corneal epithelial cells (HCECs) were stimulated with IL1β (100 ng/ml) for 24 hours. HCECs cultured in media alone served as a control. Bar chart demonstrating levels of IL36γ in the culture supernatants of indicated groups, as quantified by ELISA analysis. Data from three independent experiments are shown, and each experiment consisted of 4–6 animals/group. The values are shown as mean ± SEM (error bar). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

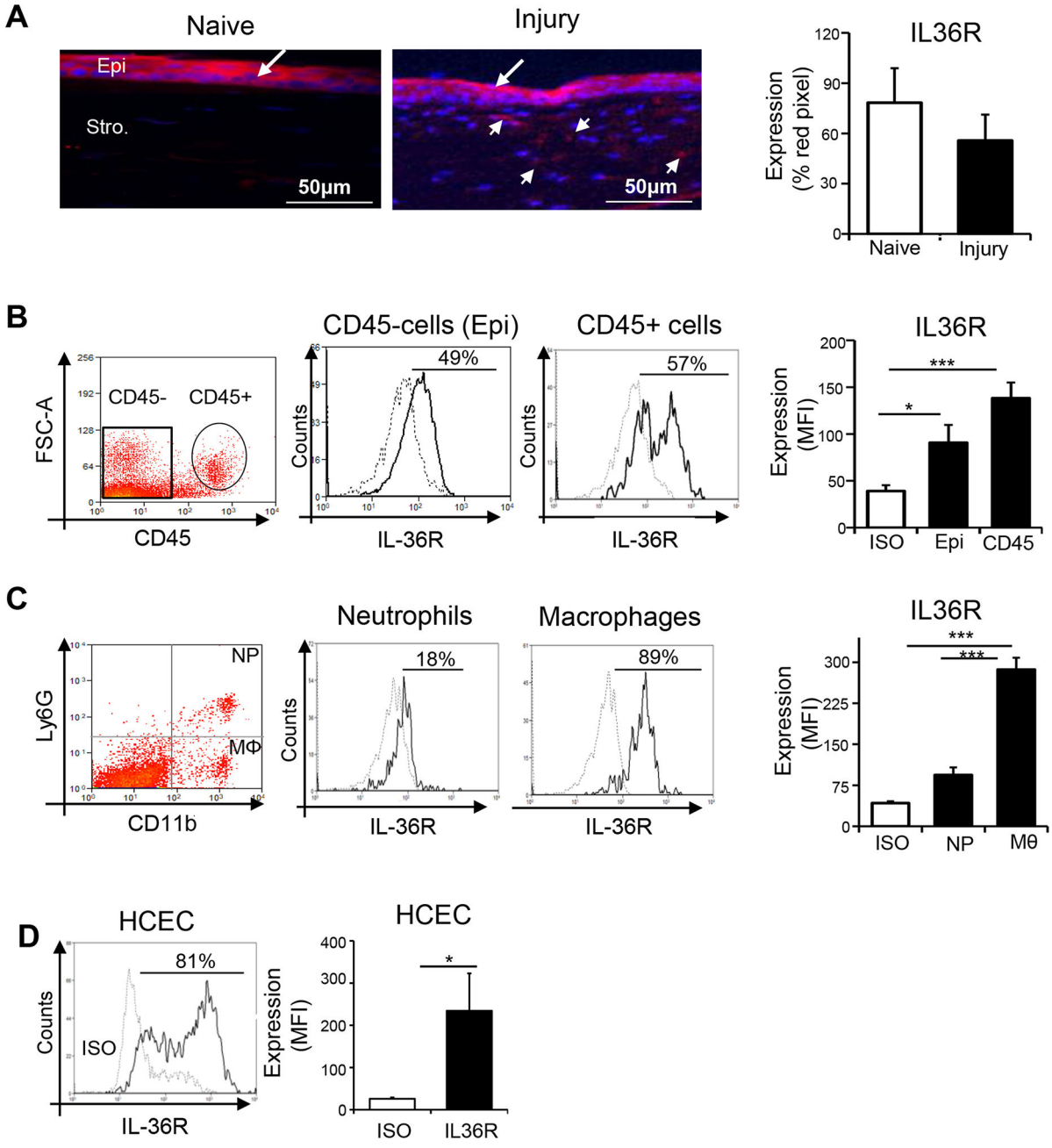

Figure 2. IL36 receptor is expressed by epithelium and immune cells at ocular surface.

Corneas were harvested from naïve mice and at 24 hrs following corneal injury. **(A)** Immunohistochemistry images of corneal cross-sections (left) and quantification bar chart (right) depicting the expression of IL36R (Red; indicated by white arrows) in corneal epithelium and stroma of naïve and injured corneas (scale bar: 50 μm). **(B)** Representative flow cytometry plots (left) showing the gating strategies and frequencies. A cumulative bar charts (right) quantifying expression (MFI; Mean Fluorescence Intensity) of IL36R by CD45− cells (primarily epithelial cells) and CD45+ leukocytes at the ocular surface. **(C)** Representative dot plots and histogram showing frequencies (left) and cumulative bar chart (right) demonstrating expression of IL36R by $CD11b^{+}Ly6G^{+}$ neutrophils (NP),

$CD11b^{+}Ly6G^{-}$ macrophages (MΦ) at the ocular surface. **(D)** Representative histogram (left) and bar chart (right) quantifying expression of IL36R on IL1β (100 ng/ml)-treated HCECs following 24 hours of culture. Data from four independent experiments are shown and each experiment consisted of four to six animals. Data are presented as mean ± SD. (error bar). *$p < 0.05$; ***$p < 0.001$.

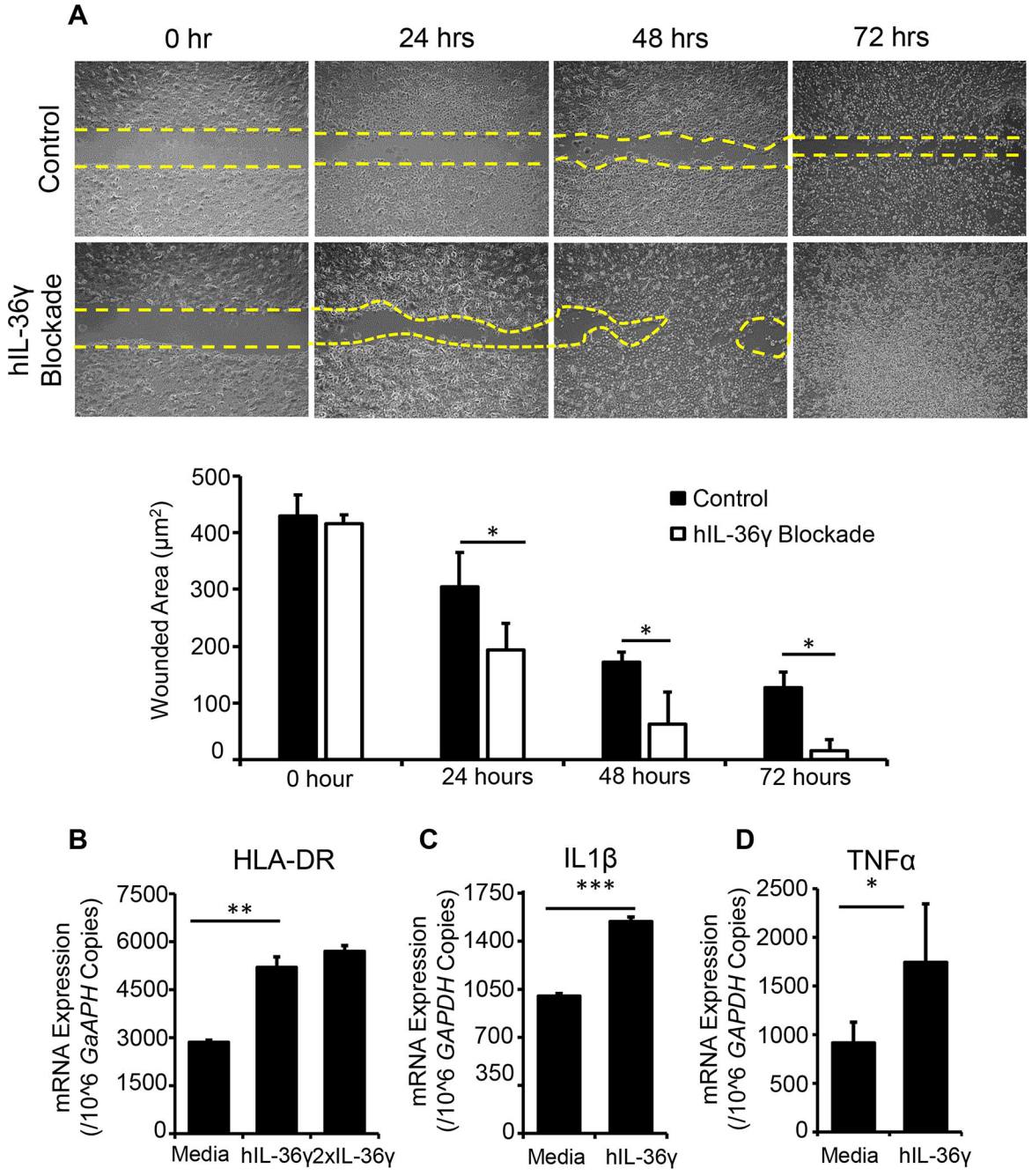

Figure 3. IL36γ delays wound closure of human corneal epithelium and promotes macrophage activation in vitro.

Human corneal epithelial cells (HCECs) were cultured in keratinocyte serum-free medium to achieve complete confluency. A wound (scratch) was created in the center of the monolayer using a pipette tip and closure of the scratch was observed in the presence of human (h) IL-36γ neutralizing antibody (100ng/ml) or isotype (control) for 72 hrs. **(A)** Representative images (upper panel) showing extent (diameter) and closure of the scratch (yellow dotted line) and cumulative bar chart quantifying area of wound (μm^2) in the monolayer of HCEC, treated with IL36γ neutralizing antibody or isotype at the indicated

time points. Total area of the wound (μm^2) was calculated from micrographs of indicated time points using ImageJ software. **(B-D)** THP1 derived macrophages were stimulated with human (h) IL36γ (100ng/ml or 200ng/ml) for 48 hrs. Real-time PCR analysis quantifying mRNA expression of **(B)** HLA-DR, **(C)** IL1β, and **(D)** TNFα (normalized to GAPDH) by macrophages following indicated treatments. Representative data from three independent experiments are shown and values are expressed as mean ± SD (error bar). Data in each group are from triplicate wells. *p < 0.05; **p < 0.01; ***p < 0.001.

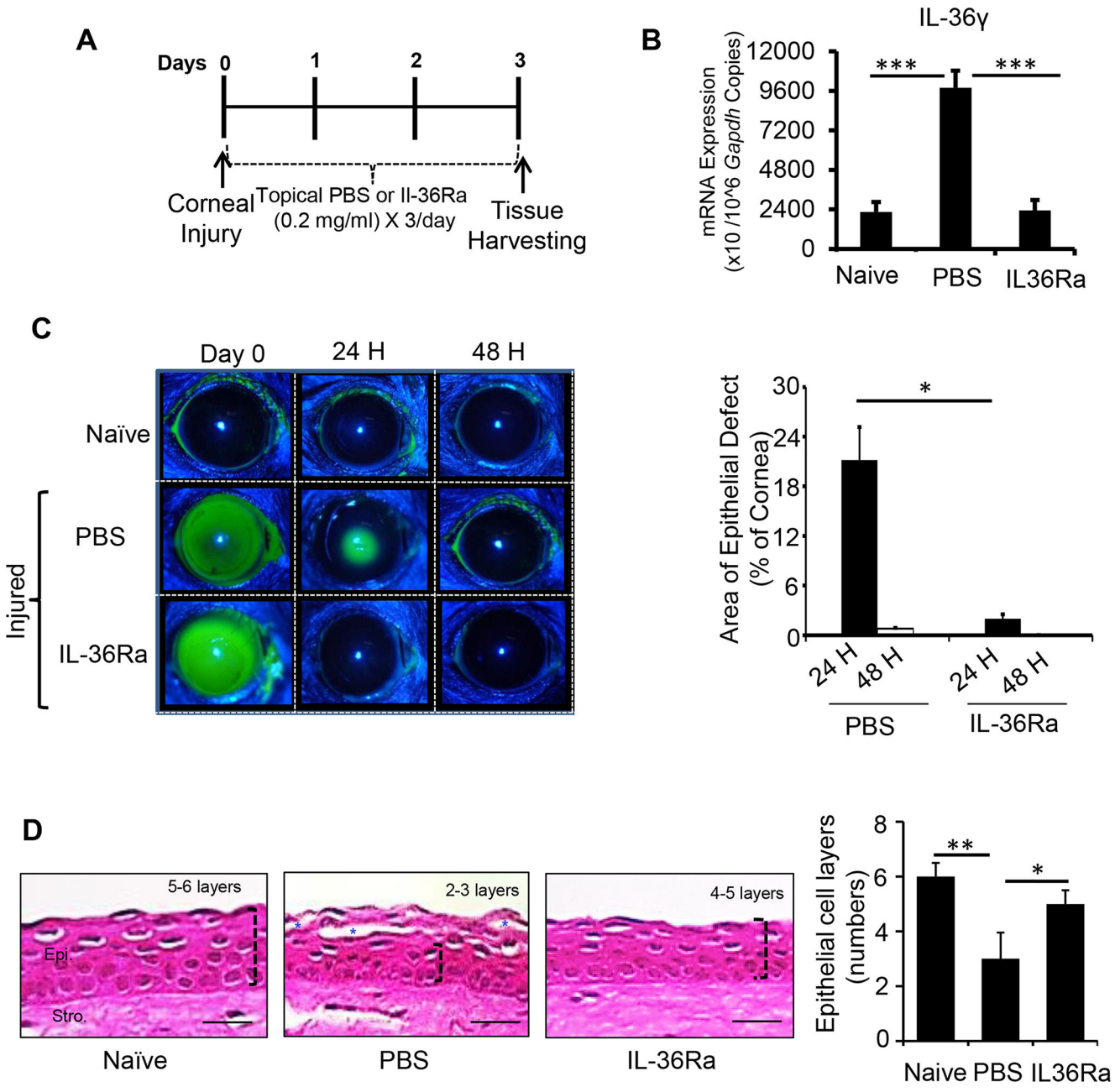

Figure 4. Blockade of IL-36γ accelerates corneal re-epithelialization in vivo.

(A) Schematics of the experimental design showing dose and frequencies of IL36Ra and PBS administration following injury. **(B)** Corneas were harvested on day 3 post-injury and IL36γ expression was evaluated using real-time PCR. **(C)** Corneal fluorescein staining of naïve and injured eyes was performed, and epithelial defects were evaluated by slit lamp biomicroscopy under cobalt blue light. The areas of epithelial defects, stained green, were quantified using ImageJ software. Representative images of fluorescein-stained corneas (left) and cumulative bar chart showing the area of epithelial defect calculated as percent increase from naïve corneas. **(D)** H&E corneal cross-sections (left) of different groups to visualize epithelial tissue structure (scale bar: 25 μm) and quantitative bar chart (right)

showing number of epithelial cell layers of indicated treatment groups. Data from at least three independent experiments are shown, and each experiment consisted of 4–6 animals/group. The values are shown as mean ± SD (error bar). *p < 0.05; **p< 0.01; ***p < 0.001. Epi., Epithelium; Stro., Stroma; blue *, disrupted epithelium.

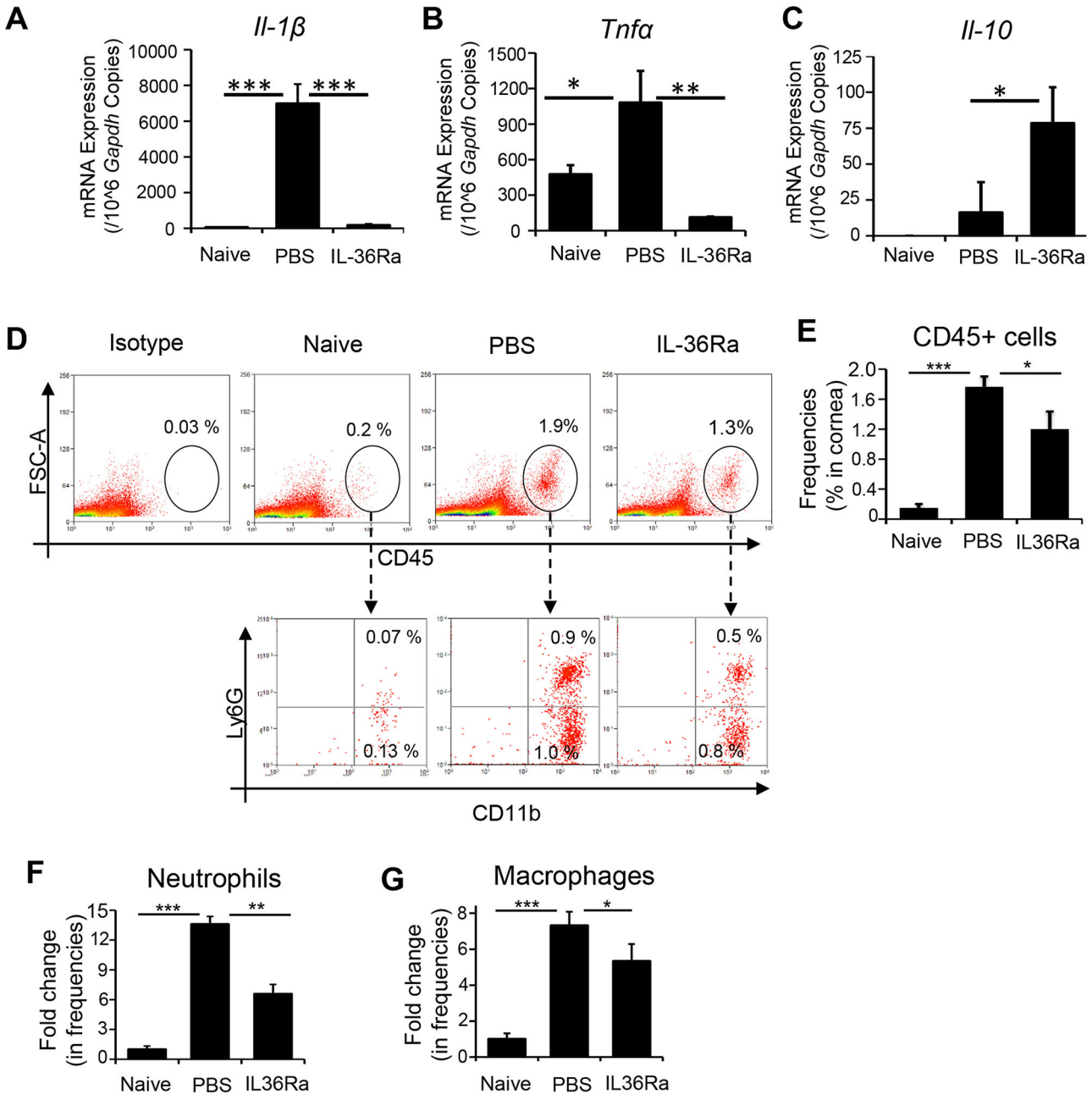

Figure 5. Blockade of Il-36γ reduces infiltration of immune cells and expression of inflammatory cytokines at the ocular surface following corneal injury.

On day 3 post-injury, corneas were harvested from naïve and injured mice treated with IL36Ra or PBS. **(A-C)** Total RNA was extracted from harvested cornea and expression of **(A)** IL1β, (**B)** TNFα, and **(C)** IL10 was assessed using real time PCR. **(D)** Single cell suspensions of corneas were analyzed for immune cell infiltration using flow cytometry. Representative dot plots showing frequencies of total CD45+ cells and gating strategies to analyze the frequencies of $CD45^{+}CD11b^{+}Ly6G^{+}$ neutrophils and $CD45^{+}CD11b^{+}$ $Ly6G^{-}$ macrophages in the corneas of naïve, PBS treated and IL36Ra treated mice. **(E)** Quantitative bar chart showing total CD45+ leukocyte infiltration (as % of total corneal cells).

Cumulative bar chart quantifying the frequencies (fold change from naïve baseline) of **(F)** Neutrophils and (**G)** Macrophages in the corneas harvested from indicated group of mice. Data from three independent experiments are shown. Each experiment consisted of 3–4 animals/group. The values are expressed as mean ± SD (error bar). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.