

Role of IL-36 γ /IL-36R Signaling in Corneal Innate Defense Against *Candida albicans* Keratitis

Chenyang Dai,^{1,2} Rao Me,¹ Nan Gao,¹ Guanyu Su,¹ Xinyi Wu,² and Fu-Shin X. Yu¹

¹Departments of Ophthalmology and Anatomy and Cell Biology Wayne State University School of Medicine, Detroit, Michigan, United States

²Department of Ophthalmology, Qilu Hospital of Shandong University, Jinan, Shandong Province, China

Correspondence: Fu-Shin X. Yu, Kresge Eye Institute, Wayne State University School of Medicine, 4717 St. Antoine Blvd, Detroit, MI 48201, USA; fyu@med.wayne.edu.

Xinyi Wu, Shandong University, Qilu Hospital, Jinan, Shandong, 250012, China; xywu8868@163.com.

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PURPOSE. Interleukin (IL)-36 cytokines have been shown to play either beneficial or detrimental roles in the infection of mucosal tissues in a pathogen-dependent manner, but their involvement in fungal keratitis remains elusive. We herein investigated their expression and function in mediating corneal innate immunity against *Candida albicans* infection.

METHODS. Gene expression in mouse corneas with or without *C. albicans* infection was determined by regular RT- and real-time (q)-PCR, Western blot analysis, ELISA or proteome profile assay. The severity of *C. albicans* keratitis was assessed using clinical scoring, bacterial counting, and myeloperoxidase (MPO) activity as an indicator of neutrophil infiltration. IL36R knockout mice and IL-33-specific siRNA were used to assess the involvement IL-33 signaling in *C. albicans*-infected corneas. B6 CD11c-DTR mice and clodronate liposomes were used to define the involvement of dendritic cells (DCs) and macrophages in IL-36R signaling and *C. albicans* keratitis, respectively.

RESULTS. IL-36 γ were up-regulated in C57BL/6 mouse corneas in response to *C. albicans* infection. IL-36 receptor-deficient mice display increased severity of keratitis, with a higher fungal load, MPO, and IL-1 β levels, and lower soluble sIL-1Ra and calprotectin levels. Exogenous IL-36 γ prevented fungal keratitis pathogenesis with lower fungal load and MPO activity, higher expression of sIL-1Ra and calprotectin, and lower expression of IL-1 β , at mRNA or protein levels. Protein array analysis revealed that the expression of IL-33 and REG3G were related to IL-36/IL36R signaling, and siRNA downregulation of IL-33 increased the severity of *C. albicans* keratitis. Depletion of dendritic cells or macrophages resulted in severe *C. albicans* keratitis and yet exhibited minimal effects on exogenous IL-36 γ -induced protection against *C. albicans* infection in B6 mouse corneas.

CONCLUSIONS. IL-36/IL36R signaling plays a protective role in fungal keratitis by promoting AMP expression and by suppressing fungal infection-induced expression of proinflammatory cytokines in a dendritic cell- and macrophage-independent manner.

Keywords: keratitis, fungal, interleukin

Fungal keratitis is an infectious keratitis that has replaced bacterial keratitis in some regions as the leading cause of corneal blindness.^{1,2} In developed countries, the main cause of fungal keratitis is the widespread use of contact lenses, with *Candida albicans* (CA) as a major pathogen. In recent years, with the abuse of antibiotics and glucocorticoids, the incidence of fungal keratitis has been increasing.³⁻⁶ Hence, studies aimed at understanding the pathogenesis of fungal keratitis are of great significance for the development of mechanism-based therapies to treat fungal keratitis.

The IL-36 family of cytokines are new members of the IL-1 superfamily and consists of three biologically similar agonists, IL-36 α , IL-36 β , and IL-36 γ , and their antagonist IL-36Ra, which is similar to IL-1Ra.⁷⁻¹⁰ IL-36 cytokines are expressed in epithelial barriers throughout the body, including skin, lung, gastrointestinal tract, and cornea.¹¹ These cytokines regulate the balance between proinflammatory and anti-inflammatory cytokine production at these tissue

sites.^{9,11} IL-36 agonists exert their effects by binding to the specific receptor IL-36R (IL-1 Receptor-Like 2, encoded by IL1RL2 gene) dimerizing with IL-1RAcP (IL-1 receptor accessory protein, encoded by IL1RAP gene), with IL-36Ra or IL-38 as antagonists.^{12,13} In addition to mucosal epithelia cells, IL-36R is constitutively expressed in dendritic cells and macrophages, two cell populations that reside in naïve corneas, and this expression increases in response to injury or infection.^{9,14} Mutations in IL-36Ra (encoded by IL36RN) are associated with the severe episodic inflammatory skin disease known as generalized pustular psoriasis.^{15,16} IL-38 is another antagonist of both IL-36R and IL-1R. Dysfunction of IL-38 expression is implicated in the pathogenesis of various autoimmune diseases such as rheumatoid arthritis, psoriasis, and systemic lupus erythematosus.¹⁷ Moreover, a significant increase in circulating and tissue levels of IL-36 α was found to be associated with primary Sjogren's syndrome.¹⁸ Collectively, these studies suggest an overall role of the IL-36 gene

TABLE. PCR Primers

sIL-1Ra	s-GGGACCCTACAGTCACCTAA	as-GGTCCTTGTAAGTACCCAGAC
IL1 β	s-TGCCACCTTTTGACAGTGATG	as-AAGGTCCACGGGAAAGACAC
IL36 α	s-CCAAGAAGTGGGGGAAATCT	as-GGAGGGCTCAGCTTTCTTTT
IL36 β	s-CATGGATCCTCACAATCTCCC	as-CTGCAAAGTTGGTTTGCCTT
IL36 γ	s-CCCATGCAAGTACCCAGAGT	as-GGGAAAGCCACTGATTCAAA
S100A8	s-TGCGATGGTGATAAAAGTGG	as-GGCCAGAAGCTCTGCTACTC
S100A9	s-GTGGCGAAAAGATCTGCAAAA	as-TCAGCTGCTTGTCTGCATTT
β -actin	s-GACGGCCAGGTCATCACTATTG	as-TCAGCTGCTTGTCTGCATTT

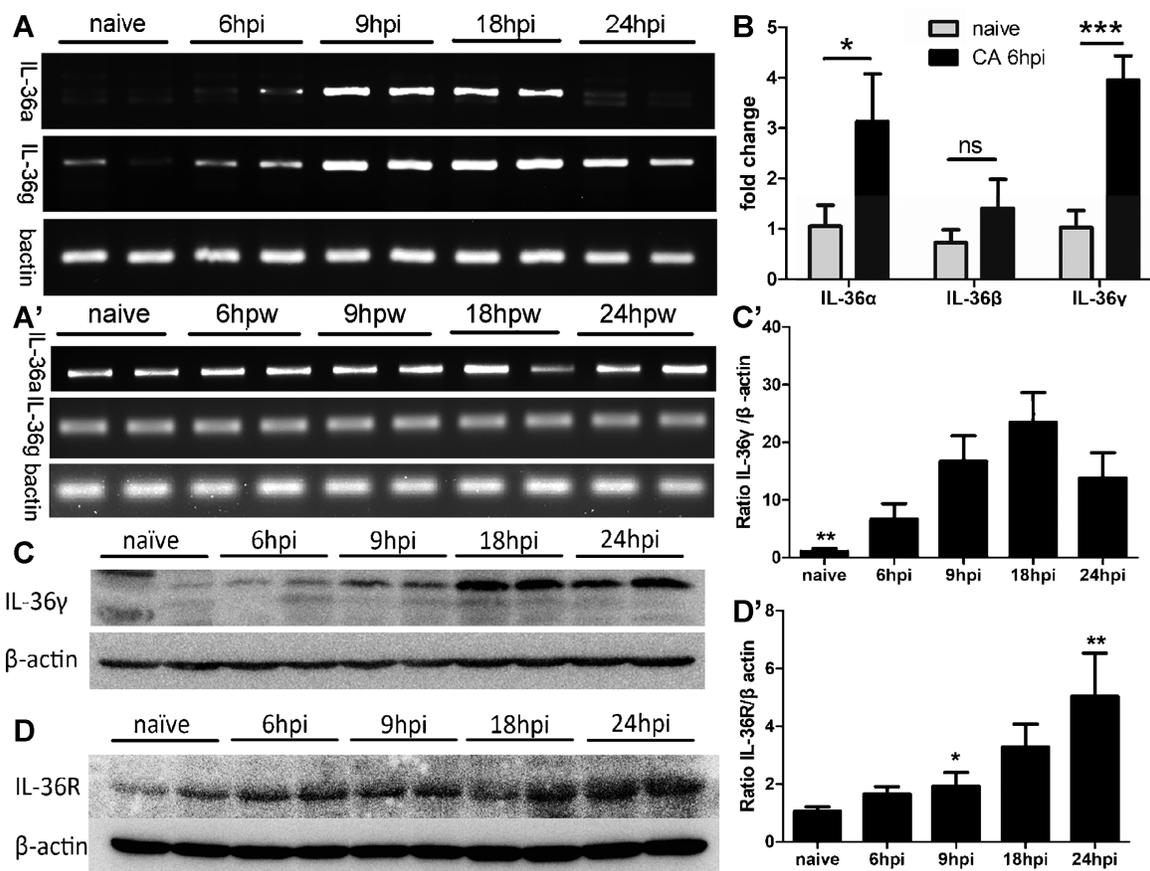


FIGURE 1. The expression of IL-36 cytokines and IL-36R in response *CA* infection in B6 mouse corneas. Mouse corneas were gently scratched with a needle, 1-mm epithelium incisions, and inoculated with 1.0×10^5 CFU *CA*. Whole corneas were collected at indicated times and subjected for RNA or protein extraction. (A) RT-PCR assessment of IL-36 α and IL-36 γ expression with uninfected naïve corneas as the controls and β -actin as internal loading controls. Two samples were used for each time point. For the sham controls, mouse corneas were scratched but not inoculated with *CA* (A'). Corneas were later collected at indicated time, followed by RT-PCR assay. (B) The qPCR analysis of the expression of IL-36 cytokines in *CA*-infected corneas at 6 hpi. Results are normalized with β -actin and presented as fold increase (mean \pm SD) relative to the control, naïve corneas, set as a value of 1. Stars on top of columns are *P* value results: **P* < 0.05, ****P* < 0.001 determined by Student's *t*-test, *N* = 3. Western blotting assessing the expression of IL-36 γ (C) and IL-36R (D) at indicated times with naïve corneas as the control. The band intensity of IL-36 γ (C') and IL-36R (D') were quantitated by densitometric analysis and the results were presented as the ratio of IL-36 γ or IL-36R versus β -actin, which served as protein loading controls. *P* values were generated using unpaired Student's *t*-test of naïve versus 6, 9, 18, or 24 hpi, ***P* < 0.01, ****P* < 0.001, *n* = 3.

family as proinflammatory cytokines in the pathogenesis of autoimmune diseases.

Because the deficiency of IL-36R antagonist appears to be associated with various autoimmune diseases,^{15,17,19,20} the underlying mechanism might be a dysfunction in controlling the basal activity of IL-36R signaling. This was supported by the finding that, unlike IL-1, IL-36 cytokines have basal expression in unchallenged tissues and cells.^{11,21} Infection has been shown to induce rapid

upregulation of IL-36 cytokines in a Toll-like receptor (TLR)-dependent manner.^{22,23} IL-36/IL-36R signaling may play a protective or detrimental role in a tissue and pathogen-dependent manner.^{10,24–29} We recently reported that soluble IL-1Ra and IL-36Ra have opposing effects on *Pseudomonas aeruginosa* (*PA*) keratitis, with IL-1Ra improving and IL-36Ra worsening the outcome of *PA* keratitis.¹¹ Our data showed that the IL-1 and IL-36 families have opposing effects in mediating innate immunity

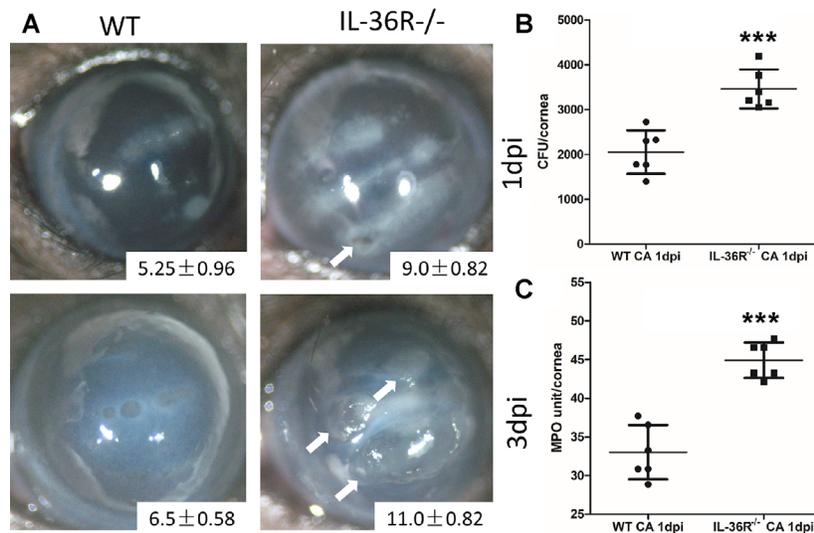


FIGURE 2. IL-36R deficiency increases the severity of CA keratitis. The centers of WT and IL-36R^{-/-} mouse corneas were gently scratched and inoculated with 1.0×10^5 cfu of CA. (A) Eyes were photographed and clinically scored at 1 and 3 dpi. The clinical scores were analyzed with nonparametric Mann-Whitney U test ($N = 5$) and presented as inserts (low right corner). $***P < 0.001$, $n = 6$. (B, C) Six WT and IL-36R^{-/-} were euthanized at 1 dpi and the corneas were excised and subjected to fungal plate counting, the results were presented as cfu CA per cornea (B) or to MPO determination, units/cornea (C). The data in B and C were presented as dot plots with the average of CFU or MPO units per cornea and error bars (mean \pm SD). P values were generated using unpaired Student's t test. ($***P < 0.001$), $N = 6$.

and inflammation. Moreover, IL-36/IL-36R signaling antagonizes IL-1/IL-1R signaling, resulting in a reduced severity of PA keratitis.¹¹ Others have reported that CA infection of the oral mucosa induces the production of IL-36 and that oral candidiasis in IL-36R^{-/-} mice shows an increased fungal burden, indicating a role of IL-36 in mediating the innate protective response to CA.¹⁰ IL-36R signaling has been shown to induce the expression of known IL-36-responsive genes encoding Il17c, and the antimicrobial peptides S100a8 and S100a9,³⁰ which form calprotectin that acts early in infection to restrict metal nutrients from CA.³¹ Calprotectin was also reported in neutrophil extracellular traps and involved in host defense against CA in vitro and in vivo.³² To date, the involvement and role of IL36 signaling in fungal keratitis remains to be determined.

In this study, we explored the function of IL-36/IL-36R signaling in mediating innate immune defense against CA infection in the corneas. We demonstrate that IL-36R activation improves the outcome of CA keratitis by inducing the expression of antimicrobial peptides and anti-inflammatory cytokines in a dendritic cell (DC)- and macrophage-independent manner. Our results suggest that IL-36 cytokines might be used as an adjunctive therapy in treating CA keratitis.

METHODS

Animals

All animal studies were approved by Wayne State University (WSU) Institutional Animal Care and Use Committee (IACUC), and all investigations conformed to the regulations of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and of the NIH. Wild-type and IL-36R^{-/-} on C57BL/6 (B6) background mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Knock-out mouse genotyping was performed by TransnetYX, Inc., Cordova, TN, USA.

Mouse Model of CA Keratitis

Mice were anesthetized by intraperitoneal injection of Ketamine (90 mg/kg) and Xylazine (10 mg/kg).³³ Corneas were scratched with a sterile 26-gauge needle to create three 1-mm incisions to break the epithelial barrier and inoculated with 1.0×10^5 CFU CA in 5 μ L PBS.

The Application of siRNA and Recombinant Protein

Recombinant IL36 γ 100 ng (R&D systems, Minneapolis, MN, USA) in 5 μ L PBS was subconjunctivally injected in each cornea at one site four hours before CA inoculation. Dharmacon SMARTpool (a mixture of 4 siRNAs) ON-TARGETplus siRNAs were used to knockdown IL-33. Mice were subconjunctivally injected twice with 5 μ L RNase-free water containing 50 pM siRNA at -24 and -6 hours before CA inoculated at 0 hpi.

CLINICAL EXAMINATION

Eyes were examined daily to monitor the disease progression with a dissection microscope and photographed. For clinical scores assessment, mice were examined at the indicated time to visually grade the disease severity. Ocular disease was graded in clinical scores ranging from 0 to 12. A total score of ≤ 5 indicated mild eye disease, 6 to 9 signaled moderate disease, and ≥ 9 severe disease.

Fungal Load Determination, Cytokine ELISA, and MPO Measurement

The corneas were excised, minced, and homogenized in 100 μ L PBS with a Qiagen Micro Tissue Grinder (Hilden, Germany). The homogenates were divided into two parts. The first part was subjected to plate counting. Aliquots (50 μ L) of serial dilutions were plated onto Salouraud agar medium plates in triplicates, and colonies were counted

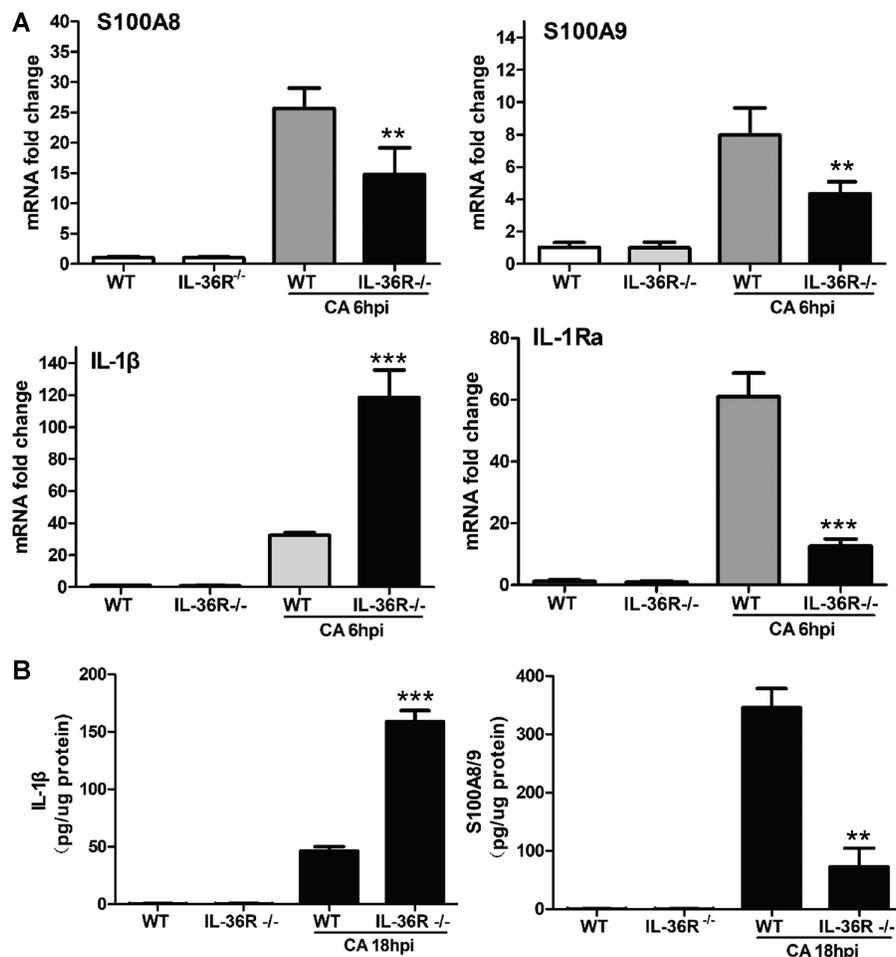


FIGURE 3. IL-36R deficiency affects the expression of calprotectin, IL-1 β , and sIL-1Ra in B6 mouse corneas in response to CA infection. WT and IL36R^{-/-} mouse corneas were inoculated with CA as in Figure 2. (A) Corneal epithelial cells were collected at 6 hpi and subjected to qPCR analysis for the expression of S100A8, A9, IL-1 β , and soluble IL-1Ra. The results of qPCR are presented as fold increase compared to the naïve, WT corneas, set as value 1 after normalization with the level of β -actin as the internal control. NS, no statistical significance. *P* values were generated using unpaired Student's *t*-test, ***P* < 0.01, ****P* < 0.001, *n* = 3. (B) Whole corneas were dissected and processed for ELISA analysis of calprotectin and IL-1 β at 1 dpi. The results of ELISA are presented as pg/ μ g total protein. *P* values were generated using unpaired Student's *t*-test, ***P* < 0.01, ****P* < 0.001, *N* = 3. UD, undetectable. Data are presented as mean \pm SD and representative of two independent experiments.

on the third day. The results were expressed as the mean number of CFU/cornea \pm standard error. Our previous study showed that by 3 dpi there was no recoverable live CA while inflammation was still lingering up to seven days³⁴; hence, we only performed fungal load determination at 1, but not 3 dpi. The second part of the homogenates was mixed with 5 μ L of 1% SDS and 10% Triton-X 100. For MPO assay, 30 μ L homogenates was mixed with 270 μ L of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0). The samples were then subjected to three freeze-thaw cycles, followed by centrifugation at 16,000g for 20 minutes. The supernatant 20 μ L was mixed with 180 μ L of 50 mM phosphate buffer (pH 6.0) containing 16.7 mg/mL O,O-dianisidine hydrochloride and 0.0005% hydrogen peroxide at a 1:30 ratio in a well of 96-well plate. The change in absorbance at 460 nm was monitored continuously for five minutes in a Synergy2 Microplate reader (BioTek, Winooski, VT, USA). The results were expressed in units of MPO activity/cornea. One unit of MPO activity corresponded to approximately 2.0×10^5 PMN. For ELISA,

protein concentration was first determined and using Micro BCA protein assay kit (Thermo Fischer Scientific, Waltham, MA, USA) and 1 μ g of total protein was used for ELISA assay for IL1 β and S100A8/9 (R&D Systems).

RNA Extraction and Real-Time PCR

For RT-PCR, mouse corneas or epithelia were collected for RNA preparation using RNeasy Mini Kit (Qiagen) followed by cDNA was generated with oligo (dT) primer and real-time PCR or regular PCR. For quantitative PCR (qPCR), cDNA was amplified using StepOnePlus Real-Time PCR system (Applied Biosystems, University Park, IL, USA) with the SYBR Green PCR Master Mix (Alkali Scientific, Fort Lauderdale, FL, USA). Data were analyzed using $\Delta\Delta$ CT method with β -actin as the internal control. RT-PCR products were subjected to electrophoresis on 2% agarose gels containing ethidium bromide. Stained gels were captured by using a digital camera. The primer pairs used for PCR were listed in the Table.

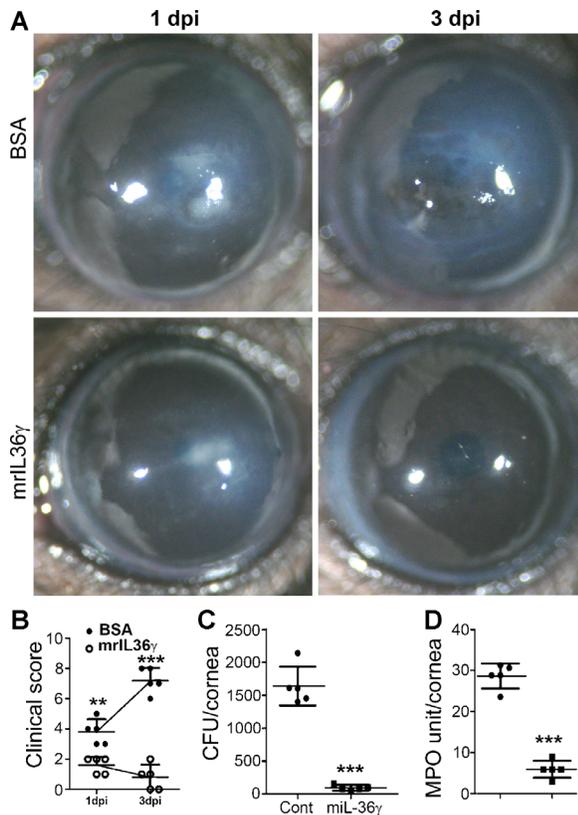


FIGURE 4. IL-36 γ exerts protective effects on CA keratitis. WT B6 mice were subconjunctivally injected with 5 μ L of IL-36 γ (100 ng/ μ L) or BSA 4 hours (–4hpi) before CA inoculation. CA inoculation was carried out as in Figure 2. (A) Representative micrographs of the control (BSA) and IL-36 γ pretreated, CA-infected corneas at 1 and 3 dpi. (B) Clinical scores of CA infected corneas pretreated with BSA (the control) and IL-36 γ were presented as mean \pm SD. $**P < 0.01$, $***P < 0.001$, determined by nonparametric Mann-Whitney U test, $n = 5$. (C) Fungal burden determined by plate counting and (D) MPO enzymatic activity of CA infected, BSA (the control) and IL-36 γ pretreated corneas at 1 dpi. The data are presented as total number of CA or MPO units per cornea and are representative of two independent experiments. The indicated P values were generated using unpaired Student's t -test, $**P < 0.01$, $***P < 0.001$, $N = 5$.

Western Blot and Mouse Proteome Profile

Mouse corneal samples were lysed with RIPA buffer and protein concentration was determined by BCA assay. For proteome profiling, 200 μ g total protein from 4 corneas in each group was detected by mouse protein array kit (R&D Systems). Another portion of the protein samples were separated by SDS-PAGE and electrically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% BSA and subsequently incubated with primary and secondary antibodies. Antibodies used: anti-mouse IL36 γ (ab233420), 36R (ab180894) from Abcam, IL-33 (R&D); anti-mouse Reg3g (Thermo Fisher Scientific); anti- β -actin (A1978; Sigma-Aldrich Corp., St. Louis, MO, USA) was used as the loading control.

Dendritic Cell and Macrophage Depletions

CD11c–diphtheria toxin (DT) receptor mice express a transgene encoding a DTR-EGFP fusion protein under the control of the murine CD11c promoter, which makes them sensitive

to DT.⁵⁵ B6-DTR mice were depleted of their DCs using 50 ng of DT in 5 μ L of PBS, administered subconjunctivally 24 hours before CA inoculation. WT B6 mice injected with 50 ng DT were used as controls. To deplete macrophages, 1 μ g of Clophosome, neutral clodronate liposomes (F70101C-N; FormuMax Scientific, Inc., Sunnyvale, CA, USA), or control liposomes (F70101-N), were subconjunctivally injected 24 hours before CA inoculation. Complete depletion of residential macrophages was observed one and two days later using immunohistochemistry with F4/80 antibody (data not shown).

Statistical Analyses

Data were presented as means \pm SD. Statistical differences among three or more groups were identified using one-way ANOVA, followed by a Bonferroni's post test to determine statistically significant differences. Analysis of clinical scores was performed by a nonparametric Mann-Whitney U test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Mouse Cornea Expresses IL-36 Cytokines During CA Infection

As a first step to understanding the role of IL-36 cytokines in CA (strain SC5314) keratitis,³⁶ we assessed the expression pattern of IL-36 cytokines and their receptor. Our previous study showed that, unlike IL-1 β and soluble IL-1Ra, IL-36R ligands have basal expression in naïve corneas. Hence, we first investigated IL-36 α and -36 γ expression using regular PCR that allows detection of the basal expression. Figure 1A shows the mRNA expression of IL-36 α and - γ in B6 mouse corneas using RT-PCR at 0, 6, 9, 18, and 24 hpi. CA infection augmented IL-36 α expression 9 hpi and 18hpi and receded at 24 hpi. IL-36 γ expression was markedly increased at 9 and 18 hpi and remained elevated at 24 hpi (Fig. 1A). In contrast, epithelial scratch wound, a sham control, did not change the DNA band intensity of IL-36 γ (Fig. 1A'). The band intensities of IL-36 α and 36 γ in Figure 1A' were the result of more cycles of PCR amplification, compared to that in Figure 1A, at corresponding times. The mRNA expression was also quantitated by real-time PCR (Fig. 1B) and the results confirmed the infection-induced expression of IL-36 α and IL-36 γ in CECs collected at 6 hpi. IL-36 α expression was elevated approximately threefold (3.14 ± 0.94) and IL-36 γ by fourfold (3.95 ± 0.48), whereas no significant increase was detected for IL-36 β . The expressions of IL-36 γ and IL-36R at the protein levels were assessed by Western blot (Figs. 1C and 1D), followed by Image J determination of pixel values normalized to that of actin (Figs. 1C' and 1D'). CA infection induced significant upregulation of IL-36 γ and IL-36R started at 9 hpi.

IL-36R Deficiency Exacerbates CA Keratitis in B6 Mice

Having shown the infection-associated upregulation of IL-36 cytokines, we next investigated the effects of IL-36R signaling on the pathogenesis of CA-keratitis, using IL-36R $^{-/-}$ B6 mice.^{14,37} At 1dpi, IL-36R deficiency increased the severity of CA keratitis, including elevated clinical scores (5.25 ± 0.96 vs. 9.0 ± 0.82 , Fig. 2A), fungal burden (2.05×10^3 vs.

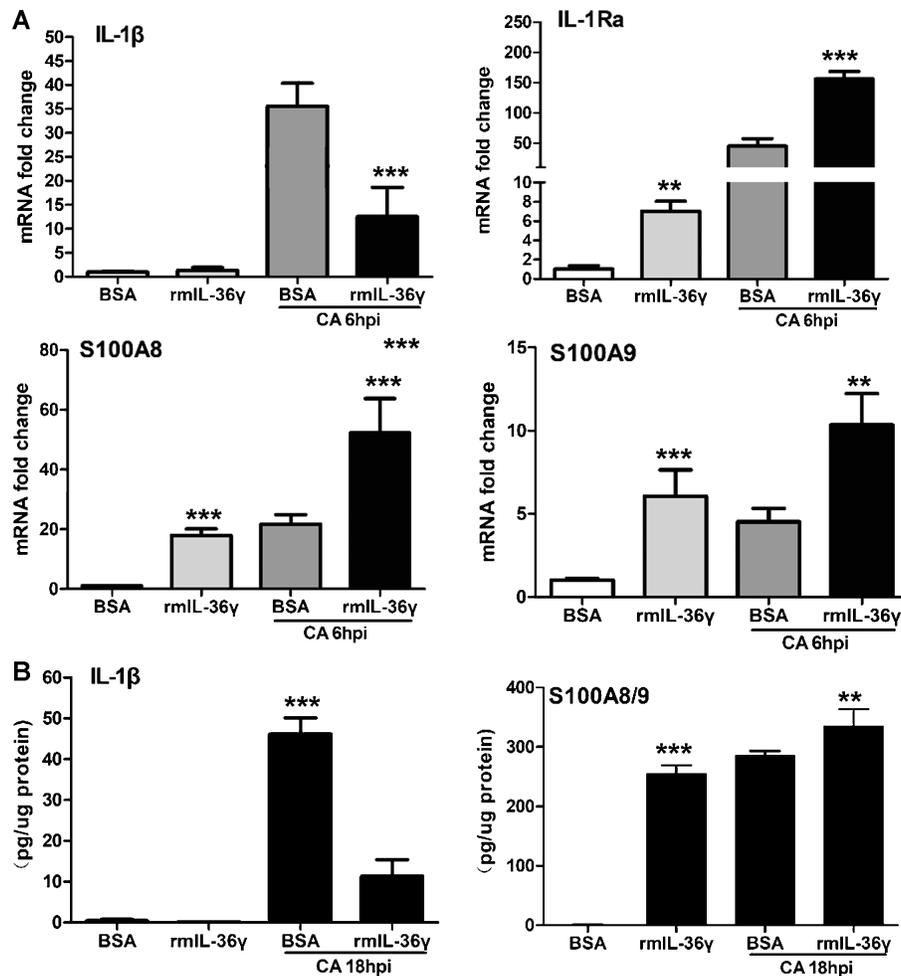


FIGURE 5. IL-36 γ differentially regulates the expression of IL-1 cytokines and calprotectin in CA infected B6 mouse corneas. WT B6 mice were subconjunctivally injected with IL-36 γ (100 ng/ μ L) or BSA 4 hours (–4 hpi) before CA inoculation as described for Figure 4. (A) The expression of IL-1 β , IL-1Ra, S100A8, and A9 in corneal epithelial cells collected at 6 hpi. The mRNA levels were assessed using qPCR analysis. The results are presented as mean of fold increase compared to naive, WT corneas, set as 1 after normalization to the level of β -actin as the internal control. (B) Whole corneas were dissected and processed at 1 dpi for ELISA analysis of calprotectin and IL-1 β . The results of ELISA are presented as pg of calprotectin and IL-1 β in 1 μ g of total protein. UD, undetectable. The *P* values were generated using unpaired Student's *t* test. BSA- and IL-36 γ -treated corneas were compared to each other as uninfected or infected at 6 hpi, respectively. ***P* < 0.01, ****P* < 0.001. Data are representative of three independent experiments with three corneas per group (mean \pm SD).

3.46×10^3 , Fig. 2B), and MPO levels (33 vs. 44.9 U/cornea, Fig. 2C), compared to the wildtype (WT) control. At 3 dpi, IL-36R-deficient mice had significantly increased keratitis severity, with higher clinical scores, 11 ± 0.82 , indicative of perforation. There was heavy infiltration into the aqueous humor and clear signs of corneal melting in IL-36R $^{-/-}$ corneas (Fig. 2A, white arrows).

IL-36R Deficiency Differentially Regulates the Expressions of IL-1 β , sIL-Ra and S100A8/A9

To investigate the mechanism of IL36/IL-36R action in CA keratitis, we collected corneal epithelia at 6hpi and whole corneas at 18hpi and assessed the levels of mRNA and protein of IL-1 β , IL-Ra, S100A8, and S100A9 of WT and IL-36 R $^{-/-}$ B6 mice by qPCR and ELISA, respectively. Figure 3A shows that in infected WT corneas, all four genes were increased to a different extent compared to the control, naive corneas (value as 1). IL-36R deficiency significantly

suppressed infection-induced expression of sIL-1Ra (61.06 vs. 12.59-fold; PCR primers were designed to amplify only soluble isoform of IL-1Ra), S100A8 (25.65 vs. 14.75 fold), and S100A9 (8.0 vs. 4.4 fold) but further augmented the expression of IL-1 β (32.53- vs. 118.6-fold). We selected IL-1 β and calprotectin (S100A8/A9) for ELISA analyses of mouse corneas (Fig. 3B). There was no detectable IL-1 β and calprotectin in the naive and IL-36R $^{-/-}$ mouse corneas; infection markedly upregulated expression of both IL-1 β (46.17 pg/ μ g total proteins) and calprotectin (345.5 pg/ μ g). IL-36R-deficiency greatly upregulated IL-1 β (158.7 vs. 46.17 pg/ μ g) but markedly downregulated calprotectin (72.51 vs. 345.5 pg/ μ g) expression in B6 mouse corneas extracted at 18 hpi.

Exogenous IL-36 γ Improves the Outcome of CA Keratitis in B6 Mouse

Having shown that proper IL-36R signaling is necessary for the corneal innate immune response to PA infection,

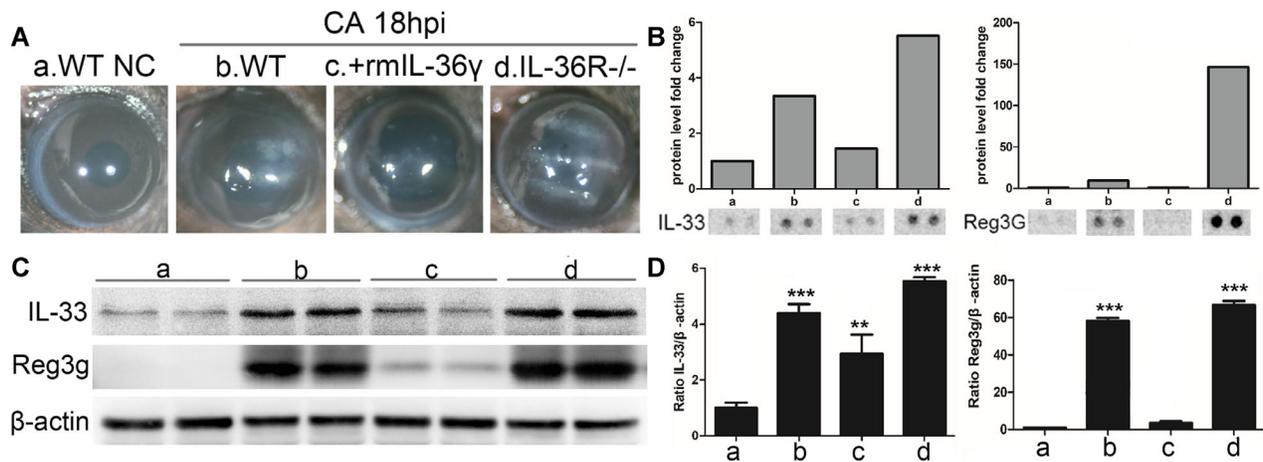


FIGURE 6. Proteome profiler analysis reveals IL-33 and REG3G as IL-36/IL-36R related genes. B6 mice were divided into four groups: (a) WT naïve, (b) WT *CA*-infected, (c) WT pretreated with rmIL-36 γ and infected with *CA*, and (d) IL-36R $^{-/-}$ infected with *CA*. (A) Micrographs of *CA*-infected corneas a–d photographed at 18 hpi. (B) Proteome Profiler Mouse XL Cytokine Array analysis of *CA*-infected corneas collected at 18 hpi. The corneas were homogenized and used for assessing cytokines with altered levels in group a–d, illustrated as *dots* for Reg3g and IL-33 (two dots per sample). The dot intensities for Reg3g and IL-33 were quantitated by densitometric analysis. (C) Western blotting of Reg3g and IL-33 in group a–d with actin staining as the internal loading control. (D) The band intensity of Western blot was quantitated by densitometric analysis and normalized with β -actin as internal control. C and D are representative of two independent experiments with two corneas per group (mean \pm SD); ** P < 0.01, *** P < 0.001 by one-way ANOVA.

we next investigated whether exogenous IL-36 γ has positive effects on ameliorating the pathogenesis of *CA* keratitis. Murine recombinant IL-36 γ (100 ng/eye) or BSA control was subconjunctivally injected four hours before *CA* inoculation. Although the infected WT corneas had typical pathological characteristics of *CA* keratitis, IL-36 γ treatment resulted in decreased opacification with lower clinical scores (Fig. 4B), lower fungal burden at 1 dpi (1.64×10^3 vs. 0.092×10^3 per cornea), and greatly reduced but still-measurable MPO activity (28.65U vs. 5.9U per cornea, Fig. 4D).

Exogenous IL-36 γ Enhances sIL-1Ra, Calprotectin While Suppresses IL-1 β Expression

To identify downstream effectors of IL-36R signaling, we next assessed the effects of exogenous IL-36 γ on the expression IL-1 β , IL-1Ra, of S100A8, and A9 (Fig. 5). In uninfected B6 mouse corneas, IL-36 γ stimulated the expression of IL-1Ra, S100A8, and S100A9 at the mRNA level (7.02-, 17.95-, and 6.06-fold, respectively). In *CA*-infected corneas, treatment with IL-36 γ downregulated IL-1 β expression (12.56- vs. 35.61-fold) while elevating the expression of S100A8 (52.2- vs. 21.73-fold), S100A9 (10.34- vs. 4.52-fold), and sIL-1Ra (156.4- vs. 45.34-fold) at 1 dpi (Fig. 5A). At the protein levels (Fig. 5B), IL-36 γ treatment resulted in a large amount of calprotectin (255.5 ± 13.7 pg/ μ g) whereas IL-1 β was undetectable in uninfected corneas. In *CA*-infected corneas, IL-36 γ markedly lowered the levels of IL-1 β (11.3 ± 4.1 from 46.2 ± 3.9 pg/ μ g) and moderately but significantly upregulated the levels of calprotectin (335.5 ± 28.2 from 286.1 ± 7.3 pg/ μ g).

Proteome Profile Assay Reveals Altered Expressions of IL-33 and REG3G in *CA*-Infected Corneas with Altered IL-36R Signaling

To further understand the mechanism of action of IL-36R signaling, we used the proteome profile assay, consist-

ing of 111 soluble mouse proteins including cytokines, chemokines, and growth factors, to identify downstream modulators in *CA*-infected corneas. Figure 6A shows the clinical phenotypes of *CA*-keratitis. As expected, exogenous IL-36 (100 ng/eye) and IL-36R deficiency decreased and increased the severity of *CA*-keratitis, respectively. Among 111 mouse proteins, staining intensities of IL-33 and Reg3g appeared to be associated with changes in IL-36 signaling: infection-increased dot intensities were suppressed by IL-36 γ and augmented in IL-36R-deficient corneas of B6 mice (Fig. 6B). Western blotting (Fig. 6C) and image analysis (Fig. 6D) confirmed the expression patterns on IL-33 and Reg3g determined by proteome profile assay. Although basal expression of IL-33 was observed in naïve corneas, Reg3g levels were undetectable. *CA* induced the upregulations of both IL-33 and Reg3g. In IL-36 γ pretreated corneas the protein levels of both IL-33 and Reg3g were lower, whereas in IL-36R-deficient mice they were higher than that of the control, infected corneas.

IL-33 siRNA Increases Severity of *CA* Keratitis

Both IL-33 and human REG3A are known to play a role in tissue inflammation and repair.^{38,39} Human REG3A (human regenerating family member 3 alpha) is a potent ROS scavenger with antioxidant and anti-gram-positive bacterium activities.⁴⁰ Mice have two homologues of human REG3A, *Reg3g* (also anti-gram-positive bacterium activity due to peptidoglycan carbohydrate binding) and *Reg3b* (anti-gram-negative bacterium by binding to lipopolysaccharide).³⁸ Hence, we focused on the role of IL-33, a member of IL-1 superfamily of cytokines, in our fungal keratitis model. Figures 7A and 7B show pathology and clinical scores of *CA* keratitis in control and IL-33-specific siRNA-treated corneas, with two micrographs for each condition. At 1 dpi, opacification was observed along the scratch line, with heavier opacification in IL-33-specific versus control, nonspecific siRNA-treated corneas. At 3 dpi, IL-33 siRNA-treated corneas had larger and denser opacification, with

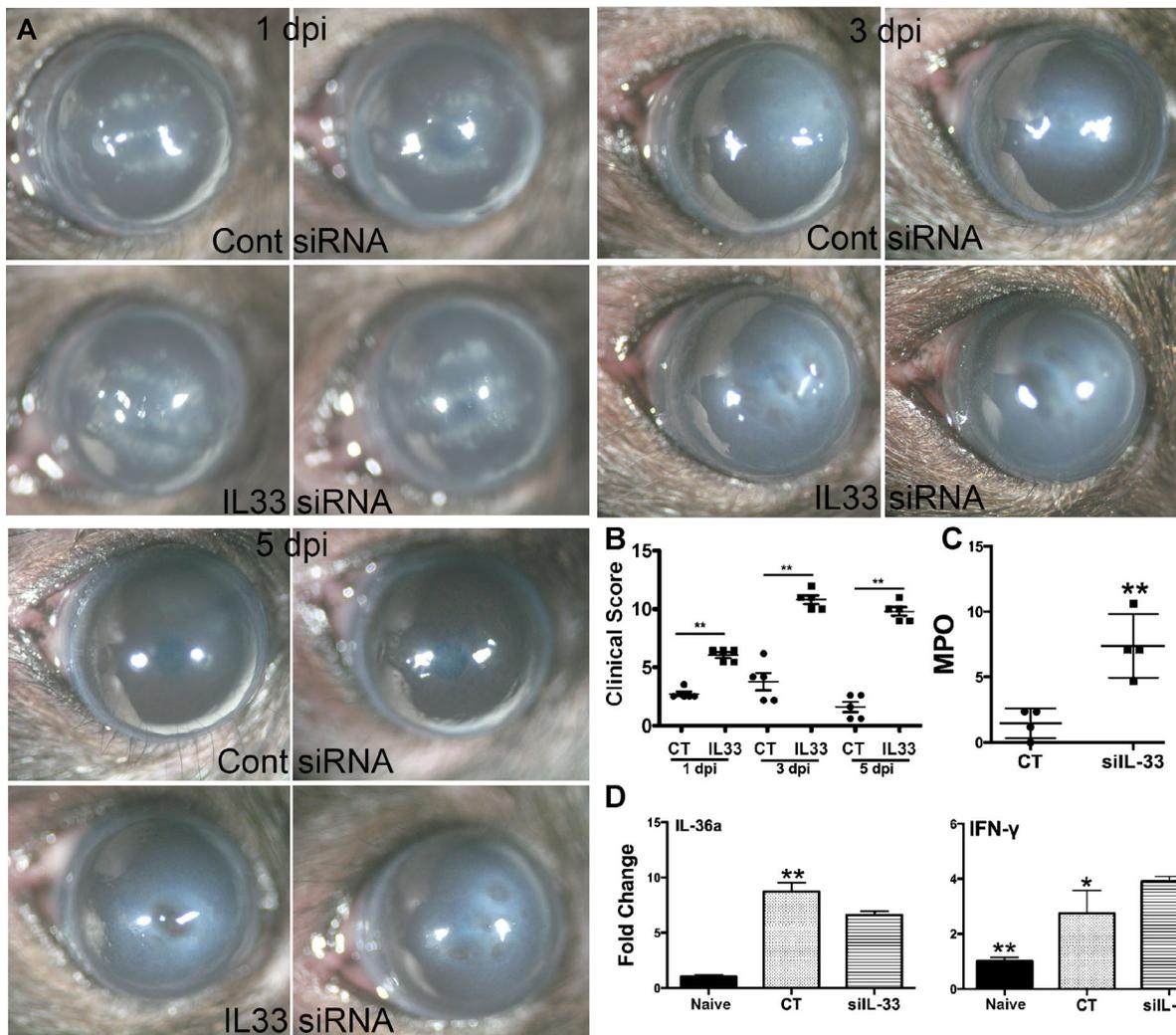


FIGURE 7. Downregulation of IL-33 increases severity of *CA* keratitis. WT B6 mice were subconjunctivally injected with IL-33 specific or the control, nonspecific siRNA (50 pmol in 5 μ L RNase free water) at -1 dpi and at -6 hpi. Corneas were scratched and inoculated with 1.0×10^5 cfu of *CA* at 0 hpi. (A) Micrographs of nonspecific siRNA (the control) and IL-33 siRNA-treated corneas photographed at indicated times, two corneas each presented. (B) Clinical scores of for the severity of keratitis were analyzed using nonparametric Mann-Whitney U test (** $P < 0.01$) at 1, 3, and 5 dpi. At 5 dpi, corneas were excised and subjected to MPO determination. This was analyzed using unpaired Student's *t*-test and presented as MPO units per cornea (C). The corneas were also subjected to qPCR analysis for the expression of IL-36 α and IFN- γ and analyzed with one-way ANOVA (D), NL, naïve cornea, $N = 5$ each, * $P < 0.05$, ** $P < 0.01$.

several regions of corneal melting, compared to the control. At 5 dpi, the control siRNA-treated corneas mostly recovered with minimal signs of inflammation, whereas IL-33 siRNA-treated corneas remained opaque with large melting areas. At 5dpi, MPO activity was significantly higher in IL-33 siRNA-treated corneas compared to control siRNA-treated corneas (Fig. 7C). Downregulation of IL-33 significantly suppressed IL-36 α while increasing IFN- γ expression in *CA*-infected corneas (Fig. 7D).

IL-36 γ Induced Innate Immune Protection is DC- and Macrophage-Independent

A previous study showed that IL-36R ligands are more potent than IL-1 β in inducing DC activation, cytokine expression, and induction of the differentiation of T-lymphocyte

into TH1 cytokine-producing cells in vitro.¹⁴ To determine whether the protective effects of IL-36R signaling on *CA* keratitis is through DCs, we depleted DCs locally through subconjunctival injection of DT into CD11c-DTR mice, with WT B6 mice receiving the same amount of DT as the control. Local depletion of DCs markedly increased the severity of *CA* keratitis, including increased corneal opacification, clinical scores (Fig. 8A), fungal load (5062 ± 303 vs. 1696 ± 287 cfu/cornea) (Fig. 8B), and MPO activity (46 ± 5 vs. 18.7 ± 3.14 units/corneas) (Fig. 8C) at 1 dpi, compared to DT-injected WT control mice. Prophylactic application of 100 ng IL-36 γ greatly suppressed the progression of *CA* keratitis with reduced clinical scores, decreased size of opacity (Fig. 8A), barely detectable levels of *CA* (16.7 ± 3.79 cfu/cornea) (Fig. 8B), and diminished MPO activity (3.81 ± 1.05 units/cornea, 12.1-fold decrease compared to DC-depleted, untreated corneas; Fig. 8C).

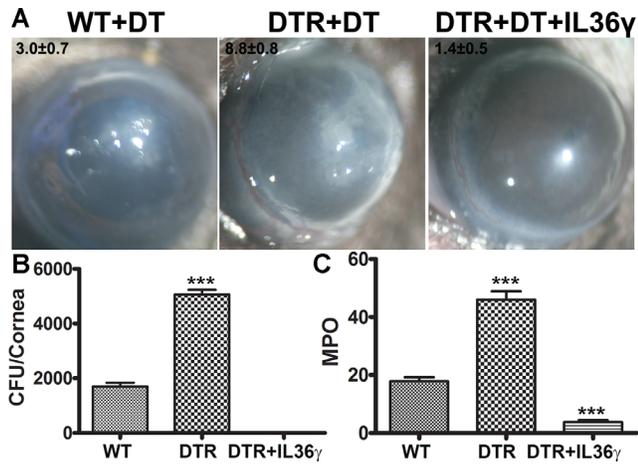


FIGURE 8. DC depletion increases the severity of but exhibits no effects on IL-36 γ -induced protection against *CA* keratitis. B6-DTR, as well as wildtype B6 mice were subconjunctivally injected with either 50 ng of DT in 5 μ L of PBS at -1 dpi, followed by injection of IL-36 γ at -4 hpi. Corneas were scratched and inoculated with 1.0×10^5 cfu of *CA* at 0 hpi. (A) Micrographs of DT treated WT B6 (the control) and B6-DTR corneas with or without exogenous IL-36 γ at 1 dpi; clinical scores were presented in the representative corneas. The diseased corneas were excised and subjected to fungal plate counting (B) and MPO determination (C) and analyzed using unpaired Student's *t*-test, $n = 5$ each, *** $P < 0.001$.

Having shown that IL-36 γ -induced protection is DC-independent, we next investigated the role of macrophages in *CA* keratitis and IL-36 γ -induced protection. Mice were treated subconjunctivally with liposomes containing clodronate to deplete corneal macrophages. Control mice were treated with liposomes containing PBS. Mice treated with PBS-liposomes exhibited similar pathology to non-pretreated corneas at 1 dpi (1695 ± 278 cfu/cornea and

17.8 ± 2.86 MPO units/cornea) and presence of IL-36 γ protected these corneas from *CA* infection (Fig. 9A) with no detectable cfu and 0.85 ± 0.05 units/cornea MPO (Figs. 9B and 9C). Mice treated with clodronate-containing liposomes had more severe keratitis than the control (Fig. 9A), with 3461 ± 575 cfu and 31.89 ± 3.51 MPO units per corneas (Figs. 9B and 9C). Prophylactic application of 100 ng IL-36 γ significantly reduced the severity of *CA* keratitis in macrophage-depleted corneas (Fig. 9A) with 308 ± 140 cfu and 8.99 ± 3.09 MPO unit per cornea, a 3.55-fold decrease compared to macrophage-depleted, untreated corneas (Figs. 9B and 9C).

DISCUSSION

We previously demonstrated that IL-1Ra and IL-36Ra have opposing effects on the outcome of *P. aeruginosa* keratitis.¹¹ In this study, we assessed the expression and functions of the IL-36 γ /IL-36R axis and observed *CA* infection-induced upregulations in expression of IL-36 α , IL-36 γ , as well as their receptor IL-36R in B6 mouse corneas. IL-36R knockout greatly exacerbated *CA* keratitis, resulting in corneal perforation at 3 dpi. Worsened outcome of *CA* keratitis in IL-36 $^{-/-}$ mice may be related to a significant upregulation of proinflammatory IL-1 β and to the suppressed expressions of anti-inflammatory sIL-1RA and antimicrobial peptides, S100A8, A9 (calprotectin). Moreover, recombinant IL-36 γ exhibited opposing effects compared to IL-36R deficiency: improved outcome of keratitis, reduced IL-1 β , and increased sIL-1RA, S100A8 and A9 expression in infected corneas. Interestingly, IL-36 γ also stimulated upregulation of calprotectin in noninfected corneas, suggesting a potential role of IL-36/IL-36R in corneal homeostasis and resistance to environmental opportunistic pathogens such as *CA*. We also showed *CA* infection induced IL-36/IL36R-related upregulation of IL-33 and Reg3g at both mRNA and protein levels. Surprisingly, downregulation of IL-33 increased the severity of *CA* keratitis,

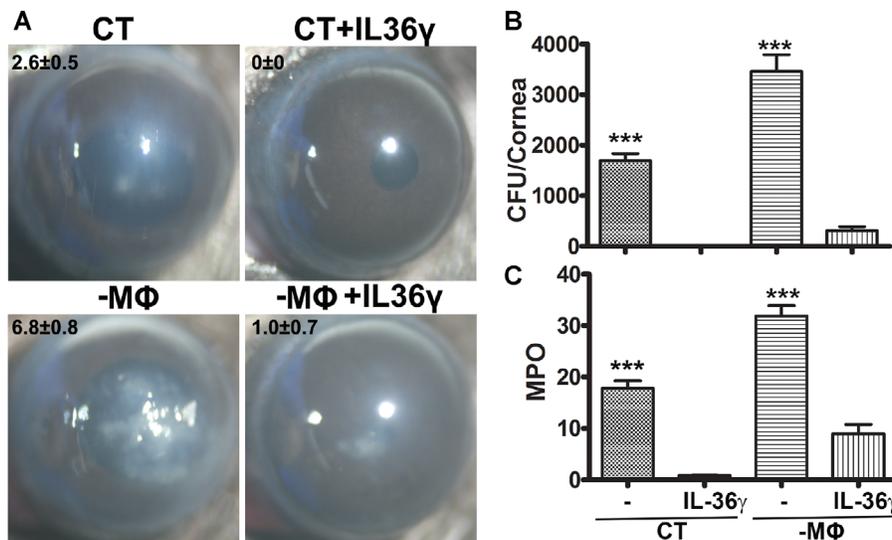


FIGURE 9. Macrophage depletion increases the severity of but exhibits minimal effects on IL-36 γ -induced protection against *CA* keratitis. B6 mice were subcutaneously injected with 1 μ g of neutral clodronate liposomes or control liposomes at -1 dpi, followed by injection of IL-36 γ at -4 hpi. Corneas were scratched and inoculated with 1.0×10^5 cfu of *CA* at 0 hpi. (A) Micrographs of liposome treated B6 corneas at 1 dpi with or without exogenous IL-36 γ ; clinical scores were presented in the representative corneas. The diseased corneas were excised and subjected to fungal plate counting (B) and MPO determination (C) and analyzed using unpaired Student's *t*-test, $n = 5$ each, *** $P < 0.001$.

suggesting a positive role of this unique member of the IL-1 family. Finally, DC and macrophage depletion greatly increased the severity but failed to overcome IL-36 γ -induced protection against CA infection of B6 mouse corneas. Taken together, our results indicate that the IL-36 γ /IL-36R axis plays a protective role in CA keratitis through upregulating AMPs and downregulating the inflammatory response, and consequently improves the outcome of CA keratitis.

IL-36 cytokines, unlike members of the IL-1 subfamily, are constitutively expressed at basal levels in mucosal epithelia, including CECs, as well as in monocytes/macrophages and T-lymphocytes.^{13,41–43} In keratinocytes, IL-36Ra was found to be constitutively expressed, whereas IL-36 α , IL-36 β and IL-36 γ were rapidly induced after stimulation with TNF α .^{44,45} Our data showed that 36 α and 36 γ were detectable by RT-PCR in naïve B6 mouse corneas and infection induced their expression. Moreover, we detected elevated expressions of IL-36 γ and IL-36R at the protein levels in a time-dependent manner in CA-infected corneas. Increased expression of IL-36R during CA infection may be due to increased infiltration of immune cells, such as dendritic cells and macrophages, but not neutrophils.⁴⁶ These results show that B6 mouse corneas increase IL-36/IL-36R signaling in response to CA infection, suggesting that IL-36 might be involved in corneal innate immunity against CA infection.

How might IL-36R mediate the innate immune response to CA infection? Various studies have shown that IL-36R activation induces the release of antimicrobial proteins such as human beta-defensins 2 and 3, LL37, S100A7, and more importantly S100A8/A9.^{30,47–49} In an oral candidiasis model, IL-36R^{-/-} mice show an increased fungal burden and reduced IL-23 gene expression.^{9,10,26,50} Previously, we found that IL-36R antagonist (IL-36Ra) downregulation and exogenous IL-36 γ prevented corneal infection while exogenous IL-36Ra augmented the pathogenesis of *P. aeruginosa*-keratitis.¹¹ Our current study provides evidence that IL-36R signaling influences the outcome of CA keratitis by suppressing the expression of proinflammatory cytokines IL-1 β and promoting the expression of IL-Ra, S100A8, and S100A9. Furthermore, our data show that IL-36R deficiency enhanced innate immune defense and reduced severity of CA keratitis. IL-36 γ -deficient mice infected with *Streptococcus pneumoniae* exhibited diminished lung bacterial clearance, increased bacterial dissemination and a higher mortality rate.^{26,51} Our results are consistent with the protective role of IL-36 γ in corneal innate defense against fungal infection. IL-36 γ significantly stimulates the expression of S100A8, S100A9, and sIL-1Ra, but not IL-1 β , in uninfected corneas in vivo. To our knowledge, this is the first report of IL-36-stimulated upregulation of S100A8/A9 or calprotectin in a mucosal surface with or without infection. Because calprotectin possesses potent anti-CA activity,^{30–32} its upregulation may represent an underlying mechanism for IL-36/IL-36R to play a protective role in CA keratitis. Although the activation of IL-1 β , a master pro-inflammatory cytokine, is most stringently regulated, its final activities are likely determined by the ratio of IL-1 β /sIL-1Ra.^{52–54} Hence, the differential effects of IL-36 γ on the expression of IL-1 β and sIL-1Ra in naïve and infected corneas is consistent with our recently-suggested notion that the IL-36 family of cytokines, as a whole, antagonize IL-1R in the corneas in response to microbial infection. Because blocking IL-1R activation has been used to treat a broad spectrum of diseases, such as gout, rheumatoid arthritis, type 2 diabetes, atherosclerosis, and acute myocardial infarction,⁵⁵ activation of IL-

36R signaling may have syngeneic effects with Anakinra, a human recombinant IL-1Ra clinically used to treat aforementioned inflammatory diseases,⁵⁶ as well as COVID-19.^{57,58}

Using a mouse proteomic array, we discovered that among others, IL-36 had a significant impact on the expression of IL-33 and Reg3 γ in CA-infected corneas. Reg3 γ is a secreted, C-type lectin and has been shown to be expressed in the cornea exposed to pathogens or after injury.^{59–61} Reg3 γ -deficient mice have increased mucosal inflammatory responses to microbiota.⁶² Compared to IL-33, a member of the IL-1 superfamily, expression of REG3 γ , an AMP, is more dramatically altered in IL-36-modulated CA-infected corneas at 1 dpi, suggesting an important role of IL-36R signaling in corneal innate immunity, as demonstrated for oral candidiasis.¹⁰ However, because murine REG3 γ is one of two homologs of human REG3A,⁶³ we focused on IL-33. The siRNA-mediated downregulation of IL-33 greatly worsened the severity of CA keratitis for up to 5 dpi, whereas the control corneas with nonspecific siRNA almost completely recovered. This result contradicts the expectation that IL-33, as a downstream effector of IL-36R signaling, would play a detrimental role in CA keratitis. Combined with the fact that the effects of IL-36R signaling on IL-33 expression were moderate, we suggest that the expression or the levels of IL-33 at 1 dpi may be related to the severity of CA keratitis rather than depending on IL-36 signaling in CA-infected corneas. Hence, IL-36 and IL-33 may function in parallel in the cornea to mediate different cellular responses to fungal infection. One potential mechanism for the observed effects of IL-33 in CA keratitis is that IL-33 acts as an alarmin cytokine to mediate innate immunity in the corneas.⁶⁴ The role and the mechanism of action of IL-33 as an alarmin or as a cytokine in innate immunity of the corneas warrant further investigation.

DCs are known to express IL-36R highly and, compared to epithelial cells, are more sensitive to IL-36 than IL-1 β .¹⁴ IL-36 was shown to stimulate DC activation and the expression of inflammatory cytokines such as IL-1 β , IL-12, IL-23, IL-6 and chemokines, including CCL1, CXCL1 and GM-CSF in an IL-36R-dependent manner.^{46,65} Surprisingly, although local depletion of DC exacerbated CA keratitis, it exhibited few, if any, effects on IL-36 γ -induced innate protection with total eradication of inoculated CA at 1 dpi. Similarly, local depletion of macrophages also resulted in severe CA keratitis and the presence of IL-36 γ protected macrophage-depleted corneas from CA infection with no detectable cfu. Comparing MPO (Fig. 8C and Fig. 9C) activities suggests that there are more neutrophils in macrophage-depleted corneas than in DC-depleted corneas. This may be due to the higher phagocytic capacity of macrophages than that of DCs in removing damaged, dead, and dying neutrophils and/or apoptotic bodies (efferocytosis).⁶⁶ The fact that depletion of both DCs and macrophages in IL-36 γ pretreated corneas results in the eradication of invading CA suggests that autocrine activation of IL-36 γ /IL-36R in epithelial cells may be the underlying cause for IL-36 γ -induced protection against CA keratitis. In IL-36 γ pretreated corneas, presence of calprotectin and other AMPs such as Reg3 γ and/or CXCL10³³ may eliminate invading CA within the epithelial layer before they penetrate the basement membrane, which functions as a barrier for pathogens to reach the stroma, as we have shown for *P. aeruginosa* infection.⁶⁷

In summary, the IL-36 family plays an important role in CA corneal infection by contributing to innate immunity in

the corneas. IL-36/IL-36R signaling alters the balance of IL-1 β and sIL-1Ra and increases the secretion of the antimicrobial peptide calprotectin and Reg3 γ . Activation of IL-36/IL-36R may be used as a means to control fungal and other causes of microbial keratitis.

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References

- Whitcher JP, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. *Bull World Health Organ.* 2001;79:214–221.
- Srinivasan M. Fungal keratitis. *Curr Opin Ophthalmol.* 2004;15:321–327.
- Bhartiya P, Daniell M, Constantinou M, Islam FM, Taylor HR. Fungal keratitis in Melbourne. *Clinical & Experimental Ophthalmology.* 2007;35:124–130.
- Bharathi MJ, Ramakrishnan R, Meenakshi R, Padmavathy S, Shivakumar C, Srinivasan M. Microbial keratitis in South India: influence of risk factors, climate, and geographical variation. *Ophthalmic Epidemiol.* 2007;14:61–69.
- Jackson BE, Wilhelmus KR, Mitchell BM. Genetically regulated filamentation contributes to *Candida albicans* virulence during corneal infection. *Microbial Pathogenesis.* 2007;42:88–93.
- Galarreta DJ, Tuft SJ, Ramsay A, Dart JK. Fungal keratitis in London: microbiological and clinical evaluation. *Cornea.* 2007;26:1082–1086.
- Mulero JJ, Pace AM, Nelken ST, et al. IL1HY1: A novel interleukin-1 receptor antagonist gene. *Biochem Biophys Res Commun.* 1999;263:702–706.
- Murrieta-Coxca JM, Rodriguez-Martinez S, Cancino-Diaz ME, Markert UR, Favaro RR, Morales-Prieto DM. IL-36 cytokines: regulators of inflammatory responses and their emerging role in immunology of reproduction. *Int J Mol Sci.* 2019;20:1649.
- Nanjo Y, Newstead MW, Aoyagi T, et al. Overlapping roles for interleukin-36 cytokines in protective host defense against murine *Legionella pneumophila* pneumonia. *Infect Immun.* 2019;87(1):e00583–18.
- Verma AH, Zafar H, Ponde NO, et al. IL-36 and IL-1/IL-17 drive immunity to oral candidiasis via parallel mechanisms. *J Immunol.* 2018;201:627–634.
- Gao N, Me R, Dai C, Seyoum B, Yu FX. Opposing effects of IL-1Ra and IL-36Ra on innate immune response to *Pseudomonas aeruginosa* infection in C57BL/6 mouse corneas. *J Immunol.* 2018;201:688–699.
- Debets R, Timans JC, Homey B, et al. Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2. *J Immunol.* 2001;167:1440–1446.
- Berglof E, Andre R, Renshaw BR, et al. IL-1Rrp2 expression and IL-1F9 (IL-1H1) actions in brain cells. *J Neuroimmunol.* 2003;139:36–43.
- Vigne S, Palmer G, Lamacchia C, et al. IL-36R ligands are potent regulators of dendritic and T cells. *Blood.* 2011;118:5813–5823.
- Onoufriadis A, Simpson MA, Pink AE, et al. Mutations in IL36RN/IL1F5 are associated with the severe episodic inflammatory skin disease known as generalized pustular psoriasis. *Am J Hum Genet.* 2011;89:432–437.
- Rajan N, Sinclair N, Nakai H, Shimomura Y, Natarajan S. A tale of two sisters: identical IL36RN mutations and discordant phenotypes. *Br J Dermatol.* 2016;174:417–420.
- Xie L, Huang Z, Li H, Liu X, Zheng S, Su W. IL-38: A New Player in Inflammatory Autoimmune Disorders. *Biomolecules.* 2019;9:345.
- Ciccia F, Accardo-Palumbo A, Alessandro R, et al. Interleukin-36alpha axis is modulated in patients with primary Sjogren's syndrome. *Clin Exp Immunol.* 2015;181:230–238.
- Takahashi T, Fujimoto N, Kabuto M, Nakanishi T, Tanaka T. Mutation analysis of IL36RN gene in Japanese patients with palmoplantar pustulosis. *J Dermatol.* 2017;44:80–83.
- Lauritano D, Ronconi G, Caraffa A, et al. New aspect of allergic contact dermatitis, an inflammatory skin disorder mediated by mast cells: can IL-38 help? *Med Hypotheses.* 2020;139:109687.
- Queen D, Ediriweera C, Liu L. Function and regulation of IL-36 signaling in inflammatory diseases and cancer development. *Front Cell Dev Biol.* 2019;7:317.
- Gresnigt MS, Rosler B, Jacobs CW, et al. The IL-36 receptor pathway regulates *Aspergillus fumigatus*-induced Th1 and Th17 responses. *Eur J Immunol.* 2013;43:416–426.
- Ahsan F, Moura-Alves P, Guhlich-Bornhof U, Klemm M, Kaufmann SH, Maertzdorf J. Role of interleukin 36 γ in host defense against tuberculosis. *J Infect Dis.* 2016;214:464–474.
- Sequeira RP, McDonald JAK, Marchesi JR, Clarke TB. Commensal Bacteroidetes protect against *Klebsiella pneumoniae* colonization and transmission through IL-36 signalling. *Nat Microbiol.* 2020;5:304–313.
- Wang P, Gamero AM, Jensen LE. IL-36 promotes antiviral immunity by boosting sensitivity to IFN-alpha/beta in IRF1 dependent and independent manners. *Nat Commun.* 2019;10:4700.
- Kovach MA, Singer B, Martinez-Colon G, et al. IL-36 γ is a crucial proximal component of protective type-1-mediated lung mucosal immunity in Gram-positive and -negative bacterial pneumonia. *Mucosal Immunol.* 2017;10:1320–1334.
- Aoyagi T, Newstead MW, Zeng X, et al. Interleukin-36 γ and IL-36 receptor signaling mediate impaired host immunity and lung injury in cytotoxic *Pseudomonas aeruginosa* pulmonary infection: role of prostaglandin E2. *PLoS Pathog.* 2017;13:e1006737.
- Aoyagi T, Newstead MW, Zeng X, Kunkel SL, Kaku M, Standiford TJ. IL-36 receptor deletion attenuates lung injury and decreases mortality in murine influenza pneumonia. *Mucosal Immunol.* 2017;10:1043–1055.
- Segueni N, Vigne S, Palmer G, et al. Limited contribution of IL-36 versus IL-1 and TNF pathways in host response to mycobacterial infection. *PLoS One.* 2015;10:e0126058.
- Hernandez-Santana YE, Leon G, St Leger D, Fallon PG, Walsh PT. Keratinocyte interleukin-36 receptor expression orchestrates psoriasiform inflammation in mice. *Life Sci Alliance.* 2020;3(4):e201900586.
- Besold AN, Gilston BA, Radin JN, et al. Role of calprotectin in withholding zinc and copper from *Candida albicans*. *Infect Immun.* 2018;86(2):e00779–17.
- Urban CF, Ermert D, Schmid M, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathog.* 2009;5:e1000639.
- Liu X, Gao N, Dong C, et al. Flagellin-induced expression of CXCL10 mediates direct fungal killing and recruitment

- of NK cells to the cornea in response to *Candida albicans* infection. *Eur J Immunol*. 2014;44:2667–2679.
34. Gao N, Liu X, Wu J, et al. CXCL10 suppression of hem- and lymph-angiogenesis in inflamed corneas through MMP13. *Angiogenesis*. 2017;20:505–518.
 35. Gao N, Yin J, Yoon GS, Mi QS, Yu FS. Dendritic cell-epithelium interplay is a determinant factor for corneal epithelial wound repair. *Am J Pathol*. 2011;179:2243–2253.
 36. Hua X, Yuan X, Mitchell BM, Lorenz MC, O'Day DM, Wilhelmus KR. Morphogenic and genetic differences between *Candida albicans* strains are associated with keratomycosis virulence. *Mol Vis*. 2009;15:1476–1484.
 37. Scheibe K, Backert I, Wirtz S, et al. IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing in vivo. *Gut*. 2017;66:823–838.
 38. Darnaud M, Dos Santos A, Gonzalez P, et al. Enteric delivery of regenerating family member 3 alpha alters the intestinal microbiota and controls inflammation in mice with colitis. *Gastroenterology*. 2018;154:1009–1023.e1014.
 39. Alves-Filho JC, Sonego F, Souto FO, et al. Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nat Med*. 2010;16:708–712.
 40. Wu Y, Quan Y, Liu Y, et al. Hyperglycaemia inhibits REG3A expression to exacerbate TLR3-mediated skin inflammation in diabetes. *Nat Commun*. 2016;7:13393.
 41. Chustz RT, Nagarkar DR, Puposki JA, et al. Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol*. 2011;45:145–153.
 42. Smith DE, Renshaw BR, Ketchum RR, Kubin M, Garka KE, Sims JE. Four new members expand the interleukin-1 superfamily. *J Biol Chem*. 2000;275:1169–1175.
 43. Bassoy EY, Towne JE, Gabay C. Regulation and function of interleukin-36 cytokines. *Immunol Rev*. 2018;281:169–178.
 44. Busfield SJ, Comrack CA, Yu G, et al. Identification and gene organization of three novel members of the IL-1 family on human chromosome 2. *Genomics*. 2000;66:213–216.
 45. Gabay C, Towne JE. Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions. *J Leukoc Biol*. 2015;97:645–652.
 46. Madonna S, Girolomoni G, Dinarello CA, Albanesi C. The significance of IL-36 hyperactivation and IL-36R targeting in psoriasis. *Int J Mol Sci*. 2019;20:3318.
 47. Johnston A, Xing X, Guzman AM, et al. IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. *J Immunol*. 2011;186:2613–2622.
 48. Botterel F, Gross K, Ibrahim-Granet O, et al. Phagocytosis of *Aspergillus fumigatus* conidia by primary nasal epithelial cells in vitro. *BMC Microbiol*. 2008;8:97.
 49. Carrier Y, Ma HL, Ramon HE, et al. Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in vivo: implications in psoriasis pathogenesis. *J Invest Dermatol*. 2011;131:2428–2437.
 50. Russell SE, Horan RM, Stefanska AM, et al. IL-36 α expression is elevated in ulcerative colitis and promotes colonic inflammation. *Mucosal Immunol*. 2016;9:1193–1204.
 51. Kovach MA, Singer BH, Newstead MW, et al. IL-36 γ is secreted in microparticles and exosomes by lung macrophages in response to bacteria and bacterial components. *J Leukoc Biol*. 2016;100:413–421.
 52. Yan C, Gao N, Sun H, et al. Targeting imbalance between IL-1 β and IL-1 receptor antagonist ameliorates delayed epithelium wound healing in diabetic mouse corneas. *Am J Pathol*. 2016;186:1466–1480.
 53. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. *Immunity*. 2013;39:1003–1018.
 54. Dinarello CA. A clinical perspective of IL-1 β as the gatekeeper of inflammation. *Eur J Immunol*. 2011;41:1203–1217.
 55. Cavalli G, Dinarello CA. Anakinra Therapy for Non-cancer Inflammatory Diseases. *Front Pharmacol*. 2018;9:1157.
 56. Ruscitti P, Berardicurti O, Cipriani P, Giacomelli R, group Ts. Benefits of anakinra versus TNF inhibitors in rheumatoid arthritis and type 2 diabetes: long-term findings from participants furtherly followed-up in the TRACK study, a multicentre, open-label, randomised, controlled trial. *Clin Exp Rheumatol*. 2021;39:403–406.
 57. Kyriazopoulou E, Panagopoulos P, Metallidis S, et al. An open label trial of anakinra to prevent respiratory failure in COVID-19. *eLife*. 2021;10:e66125.
 58. Pontali E, Volpi S, Signori A, et al. Efficacy of early anti-inflammatory treatment with high doses of intravenous anakinra with or without glucocorticoids in patients with severe COVID-19 pneumonia. *J Allergy Clin Immunol*. 2021;147:1217–1225.
 59. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol*. 2010;10:131–144.
 60. Liu X, Zhou Z, Cheng Q, et al. Acceleration of pancreatic tumorigenesis under immunosuppressive microenvironment induced by Reg3g overexpression. *Cell Death Dis*. 2017;8:e3033.
 61. Mirabelli P, Mukwaya A, Lennikov A, et al. Genome-wide expression differences in anti-Vegf and dexamethasone treatment of inflammatory angiogenesis in the rat cornea. *Sci Rep*. 2017;7:7616.
 62. Loonen LM, Stolte EH, Jaklofsky MT, et al. REG3 γ -deficient mice have altered mucus distribution and increased mucosal inflammatory responses to the microbiota and enteric pathogens in the ileum. *Mucosal Immunol*. 2014;7:939–947.
 63. Choi SM, McAleer JP, Zheng M, et al. Innate Stat3-mediated induction of the antimicrobial protein Reg3 γ is required for host defense against MRSA pneumonia. *J Exp Med*. 2013;210:551–561.
 64. Cayrol C, Girard JP. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr Opin Immunol*. 2014;31C:31–37.
 65. Foster AM, Baliwag J, Chen CS, et al. IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin. *J Immunol*. 2014;192:6053–6061.
 66. Boada-Romero E, Martinez J, Heckmann BL, Green DR. The clearance of dead cells by efferocytosis. *Nat Rev Mol Cell Biol*. 2020;21:398–414.
 67. Gao N, Kumar A, Yu FS. Matrix metalloproteinase-13 as a target for suppressing corneal ulceration caused by *Pseudomonas aeruginosa* infection. *J Infect Dis*. 2015;212:116–127.