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Lacrimal gland-derived IL-22 regulates IL-17-mediated ocular mucosal inflammation

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Abstract

Inflammatory damage of mucosal surface of the eye is a hallmark of dry eye disease (DED), and in severe cases can lead to significant discomfort, visual impairment, and blindness. DED is a multifactorial autoimmune disorder with a largely unknown pathogenesis. Using a cross-sectional patient study and a well-characterized murine model of DED, herein we investigated the immunoregulatory function of interleukin-22 (IL-22) in the pathogenesis of DED. We found that IL-22 levels were elevated in lacrimal fluids of DED patients and inversely correlated with severity of disease. Acinar cells of the lacrimal glands, not inflammatory immune cells, are the primary

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AUTHOR CONTRIBUTIONS

YWJ designed the study; collected the patients' tear samples, performed animal experiments, and wrote the manuscript. SKM assisted in designing experiments, analyzed the data, and wrote the manuscript. HSH collected patients' tear samples. EJC performed LC-MS/MS experiments and assisted in molecular experiments. JHL helped to design and conceive of the *in vivo* study. YS performed immunostaining experiments. AY performed flow cytometry and molecular experiments. HN managed and generated all animal models. HSL assisted in statistical analysis of data. SKC and HKL designed the study, analyzed data, interpreted the results, wrote and reviewed this manuscript.

DISCLOSURES

Authors have no conflict of interests to declare.

source of IL-22, which suppresses inflammation in ocular surface epithelial cells upon desiccating stress. Moreover, loss of function analyses using IL-22 knock-out mice demonstrated that IL-22 is essential for suppression of ocular surface infiltration of Th17 cells and inhibition of DED induction. Our novel findings elucidate immunoregulatory function of lacrimal gland-derived IL-22 in inhibiting IL-17-mediated ocular surface epitheliopathy in DED thus making IL-22 a new relevant therapeutic target.

INTRODUCTION

Ocular mucosal inflammation is characteristic for dry eye disease (DED),[1, 2] a highly prevalent ocular surface autoimmune disorder. Uncontrolled severe DED can lead to vision loss due to inflammation-induced corneal ulceration and scaring.[3, 4] In the progression of DED, pathogenic immune cells, predominantly Th17 cells continuously migrate to the ocular mucosal surface[5–9] and secrete pro-inflammatory mediators, including interleukin (IL)-17,[10, 11] IL-1,[12] and tumor necrosis factor (TNF)-α[13, 14] causing ocular surface inflammation and epitheliopathy. Despite strong evidence that Th17 cells are involved in ongoing DED inflammation, the precise pathological and regulatory mechanisms are not yet well understood.

IL-22, a member of the IL-10 superfamily, is a multifunction cytokine which is secreted by various immune cells such as Th17 and innate lymphoid cells during the course of mucosal immune response.[15] Unlike other interleukins, IL-22 does not directly regulate the function of immune cells. Instead, it binds to the IL-22 receptor (IL-22R) on cells of the skin, pancreas, liver, kidney, and tissues of the digestive and respiratory systems.[15–18] Serum levels of IL-22 have been shown to be significantly elevated in patients with Sjögren's syndrome (SS), an autoimmune disorder causing dry eye and dry mouth conditions.[19] Both protein and mRNA levels of IL-22, IL-23, and IL-17 have also been reported significantly high in the inflammatory salivary glands of SS patients.[20] In addition, phosphorylation of signal transducer and activator of transcription 3 (STAT3), a specific transcriptional activator of IL-22 and IL-17 is up-regulated in the exocrine glands of SS patients[21, 22] suggesting a functional association between IL-22 and IL-17 in the pathogenesis of SS. Recently, elevated levels of IL-22 have been shown in tears of patents with DED.[11] Moreover, IL-22 has been shown to promote epithelial wound healing.[23] Despite multiple reports on expression of IL-22 in various eye conditions, the precise function of IL-22 in the immunopathogenesis of DED remains unknown.

In the present study, we have investigated the function and cellular source of IL-22 in the pathogenesis of DED. We found, for the first time, that the lacrimal gland secretes elevated levels of IL-22, which prevents ocular surface epitheliopathy and infiltration of pathogenic Th17 cells. Using IL-22 deficient mice we confirmed that IL-22 contributes to the inhibition of Th17-medited inflammation, and suppresses initiation and development of DED.

RESULTS

Increased IL-22 levels in lacrimal fluids of patients with dry eye disease

DED pathogenesis is primarily associated with the infiltration of Th17 cells, which have been demonstrated to secrete IL-17 and IL-22.[11, 16] Since tears are a mixed fluid composed of secretions from the lacrimal gland (LG), meibomian gland, and corneoconjunctival cells, we precisely collected the pure LG-secreted fluid (LF) and total tear fluid (TF) from conjunctival sac (Supplement 1, video file) of healthy controls and DED patients to evaluate the expression level of IL-22 and IL-17. IL-22 levels in LFs of DED patients (median 1621 pg/ml; interquartile, IQR 1154 – 2641) were significantly higher as compared to the healthy control (median 51 pg/ml, IQR 25 – 99, $p < 0.001$; Fig 1A). However, no significant difference in the IL-22 levels was observed in TFs of DED patients (median 566 pg/ml, IQR 421 – 811) and healthy controls (median 492 pg/ml, IQR 403 – 621). These increased levels of IL-22 in LFs but not TFs, suggest that LGs could be primary source of high levels of IL-22 in DED. IL-17 levels in LFs (median 769 pg/ml, IQR 421 – 1101) of DED patient were significantly higher as compared to healthy controls (median 27.5 pg/ml, IQR 19 – 45, $p < 0.001$; Fig. 1B). In contrast to IL-22, IL-17 levels in TFs of DED patients (median 3289.5 pg/ml, IQR 2514 – 3688) were higher than in healthy controls (median 527 pg/ml, IQR 216 – 621, $p < 0.001$).

IL-22 levels inversely correlate with the severity of dry eye disease

Having shown the increased expression of IL-22 and IL-17 in the LFs of DED patients, we next determined the association of IL-22 and IL-17 levels with the severity of DED. Correlation and regression analyses were performed to determine correlation between cytokine levels and the severity of DED measured as ocular surface disease index (OSDI). Significant negative correlations between the levels of IL-22 both in LFs ($r = -0.777$, $p < 0.001$) or TFs ($r = -0.922$, $p < 0.001$) and OSDI score were observed suggesting that IL-22 at the ocular surface could be associated with reduction of disease severity (Fig 1C). In contrast to IL-22, significant positive correlations between the IL-17 levels both in LFs ($r = 0.869$, $p < 0.001$) or TFs ($r = 0.855$, $p < 0.001$) and OSDI scores was observed indicating that IL-17 is involved in augmenting disease severity (Fig 1D). Moreover, OSDI scores below 20 were associated with undetectable levels of IL-17 in both LFs and TFs.

Lacrimal glands expressed high levels of IL-22 in a murine model of dry eye disease

Increased IL-22 expression particularly in LFs and its negative correlation with disease severity prompted us to examine the cellular source of IL-22 in DED. Since investigating the source of IL-22 in human LGs was not possible, we used a well standardized murine model of DED[5–8] for further investigations. LGs from DED and healthy mice (control) were harvested. Real-time PCR was performed to measure IL-22 mRNA expression. Similar to our observation in DED patients, DED induction in mice resulted in a significant increase in IL-22 mRNA expression compared to control non DED mice (Fig 2A). IL-22 expression was further confirmed at protein level by performing ELISA on the supernatants from LG lysates. A significant 5 – 6 fold increase in IL-22 protein was observed in DED mice compared to control group (Fig 2B). Furthermore, we investigated the precise cellular source of IL-22 in the LG using immunohistochemistry. DED induction resulted in increased IL-22

expression and massive LG infiltration of leukocytes compared to control mice. But surprisingly, this increased IL-22 expression was associated primarily with the glandular acinar cells, and not infiltrating leukocytes (Fig 2C, D). These IL-22 positive acinar cells were in close proximity of infiltrating immune cells.

IL-22 receptor expression on the ocular surface

The fact that receptor expression at the site of action is essential for cytokines to exert their function led us to investigate the IL-22 receptor system at the ocular surface. IL-22 receptor is a heterodimer of IL-22R1 and IL-10R2 subunits. Real time PCR for IL-22R1 and IL10R2 mRNA expression in total corneal and conjunctival lysates was performed. The results revealed that IL-22R1 is constitutively expressed at the ocular surface and its expression does not alter after DED induction. However, IL-10R2 expression was upregulated at the ocular surface in DED mice compared to control mice (Fig 3A). Similar to mRNA expression, protein expression analyses using flow cytometry and immunohistochemistry revealed that both IL-22R1 and IL-10R2 are expressed at the ocular surface (Fig 3B, C), and IL-10R2 was increased in mice with DED compared to healthy control.

IL-22 inhibits the expression of inflammatory cytokines by ocular surface epithelial cells under hyperosmolar stress

Because IL-22 expression is increased in LGs in DED, and its receptor is expressed on the ocular surface, we next examined the effect of IL-22 on the production of inflammatory cytokines by corneal epithelial cells *in vitro* under desiccating hyperosmolar stress. This assay mimics the pathophysiology of desiccating stress-induced ocular surface inflammation in DED.[24] Primary human corneal epithelial cells (hCECs) were exposed to 380m osmolar medium to induce hyperosmolar stress in the presence and absence of IL-22. After 18 hours, culture supernatants were collected and ELISA was performed to assess the expression of inflammatory cytokines IL-1β, TNF-α, and, IL-8 (Fig 4A), and the Th17-inducer cytokines IL-6 and IL-23 (Fig 4B). Hyperosmolar stress induced the secretion of IL-1β, TNF-α, IL-8, IL-6 and IL-23 by hCECs, and this induction could be substantially suppressed by IL-22. These results indicate that IL-22 exerts anti-inflammatory effects on ocular surface epithelial cells.

IL-22 deficiency enhances ocular surface infiltration of IL-17 secreting cells and epitheliopathy in dry eye disease

Finally, to determine the function of IL-22 in the pathogenesis of DED *in vivo*, we created IL-22 deficiency either using an IL-22 neutralizing antibody (αIL-22) or IL-22 knock-out (KO) mice. αIL-22s were intravenously administered into mice during DED induction. PBS injected mice served as controls. Seven days after disease induction, ocular surface tissues (cornea and conjunctiva) were harvested to measure the frequencies of IL-17 secreting cells using flow cytometry. DED induction resulted in increased infiltration of IL-17 secreting cells, which was further augmented in αIL-22 treated mice as compared to PBS control (Fig 5A). IL-22 function in DED pathogenesis was further confirmed using genetically deficient IL-22 KO mice. DED was induced in IL-22 KO and wild type (WT) mice. Then frequencies of IL-17 secreting cells on the ocular surface and progression of disease by grading corneal epithelial erosions were evaluated. Consistent with blocking IL-22 *in vivo*, IL-22 KO mice

showed significantly enhanced ocular surface infiltration of IL-17 positive cells compared to WT mice (Fig 5B). Regarding the cellular origin of increased IL-17 secretion at ocular surface in DED mice, flow cytometric analysis of ocular surface tissues revealed increased infiltration of CD4+IL-17+ cells (Th17; 14.00 %), but not CD3ε-NK1.1+IL-17+ cells (NK cell; 1.67 %) and CD11b+Gr-1+IL-17+ cells (Neutrophils; 1.93%), (Supplement 2). Using Immunohistochemistry, effect of IL22 on infiltration of IL-17 secreting CD4 cell was further confirmed in IL-22 KO mice after DED induction. Consistent with flow cytometry analysis, IL-22 deficiency resulted in increased infiltration of CD4+IL-17+ cells in conjunctival cross sections after DED induction (Fig. 5C, white arrows). Moreover, most of CD4+IL-17+ primarily infiltrated into conjunctival epithelia (Fig. 5C, green arrows). As IL-6 and IL-23 are major cytokines that promote Th17 immune response,[25–28] we next estimated ocular surface levels of IL-6 and IL-23 in WT and IL-22 KO mice after DED induction. ELISA analysis on corneoconjunctival lysates showed elevated levels of IL-6 and IL-23 in WT DED mice compared to the normal control group. Similar to Th17 frequencies, IL-22 KO mice showed a significant 2.5 fold increase in the ocular surface level of IL-6 and IL-23 compared to WT after DED induction (Fig. 5D). We next investigated the effect of IL-22 epitheliopathy by monitoring corneal erosions, which is a pathological characteristic of DED, in both WT and IL-22 KO mice. IL-22 KO mice showed a significant 3-fold increase of the corneal erosion grade during the early induction phase (day 1 and 3) compared to WT mice (Fig. 5E). However, in the later stage of the disease (day 7 onward) the corneal erosion scores in IL-22 KO and WT mice were similar. Having shown earlier that non immune acinar cells are a major source of IL-22, we next investigated whether IL-22 secretion by non-immune acinar cells is critical for the suppression of pathogenic Th17 cell responses during DED progression. Bone marrow (BM) chimera experiments were performed where WT and IL-22 KO irradiated mice were reconstituted with BM cells of IL-22 KO and WT mice, respectively (Fig. 6A). At 7 days post-DED induction, flow cytometry analysis of corneoconjunctival cells revealed increased infiltration of ocular surface Th17 cells in the IL-22 KO host with WT BM compared to the WT host with IL-22 KO BM (Fig. 6B). Consistently, the cornea erosion grades were higher for the IL-22 KO host with WT BM than the WT host with IL-22 KO BM during DED (Fig. 6C).

Collectively, these results suggest that non immune cell derived IL-22 is essential for the suppression of pathogenic Th17 infiltration after DED induction.

DISCUSSION

This study demonstrates the immunoregulatory function of IL-22 in suppressing IL-17-mediated ocular mucosal inflammation in DED. Specifically, we show herein that elevated secretion of IL-22 by the LG in DED patients negatively correlates with disease severity. Acinar cells of the LGs, and not infiltrating immune cells, produce IL-22, which protects ocular surface epithelium and inhibits infiltration of Th17 cells to the ocular surface in desiccating stress-induced DED.

In the pathogenesis of DED, T cell cytokines, primarily IL-17 initiate the immune response and cause inflammation at the ocular surface.[6, 11, 29] In addition to IL-17, IL-22 levels have also been reported to be increased in many autoimmune disorders.[16, 30] We show that LF

from DED patients has increased levels of IL-17 and IL-22, two Th17 cytokines which have been associated with the pathogenesis of various autoimmune disorders, including Sjögren's syndrome. In accord with previous studies,[11, 31] we observed that IL-17 levels positively correlate with disease severity. However, our study further shows that IL-22 levels in tears inversely correlate with severe disease symptoms, suggesting a protective role of IL-22 in the DED. Since LF is diluted with secretions from meibomian glands and corneoconjunctival cells while constituting the TF at the ocular surface, we observed a moderate increase in IL-22 levels in TF of DED patients compare to healthy controls. IL-22 has been strongly associated with mucosal immune response due to its secretion by immune cells and direct effect on mucosal surface of various organs such as pancreas, liver, kidney, and joints.[18, 32] Because it is difficult to examine the exact cellular source of IL-22 in human subjects, we used a well characterized mouse model of DED to explore the source and precise function of IL-22 in the pathogenesis of DED. Multiple reports suggest that IL-22 is primarily secreted by Th17, Th22, NK cells, and innate lymphoid cells.[15, 33, 34] Our study provides novel evidence that non-immune cells such as acinar cells in the LGs secrete IL-22, and this secretion is augmented in DED condition. Moreover, results from BM chimera experiments suggest protective function of non-immune cells secreting IL-22 in DED (Fig. 6).

We demonstrate an increased expression of IL-22 receptors (heterodimer of IL-22R1 and IL-10R2) by the ocular surface epithelium, indicating the importance of an IL-22 mediated signaling cascade in the immunopathogenesis of DED. Expression of IL-22 receptors has been shown previously on other non-ocular mucosal surfaces such as lung and intestine.[35, 36] DED is associated with the increased expression of proinflammatory cytokines TNF-α, IL-1β, IL-8, and Th17-inducing cytokines IL-6 and IL-23 at the ocular surface.[5] Here, we show that IL-22 inhibits expression of such inflammatory cytokines and Th17 inducing cytokines IL-6 and IL-23 by ocular surface epithelial cells under desiccating (hyperosmotic) stress.

Utilizing the loss of function approach, we finally determined the *in vivo* contribution of IL-22 in the pathogenies of DED. We blocked the endogenous IL-22 using a neutralizing antibody in a mouse model of DED. Our study shows that *in vivo* neutralization of IL-22 led to increased infiltration of IL-17-secreting cells at the ocular surface under desiccating stress suggesting IL-22 is essential for inhibiting Th17-mediated mucosal inflammation in DED. We confirmed the effect of IL-22 on Th17 cell infiltration and progression of DED pathogenesis using IL-22 KO mice. Consistent with our IL-22 neutralization assay, IL-22 KO mice with DED showed a profound infiltration of IL-17 positive cells to the ocular surface. Based on our previous results showing IL-22 mediated inhibition of expression of Th17 inducers IL-6 and IL-23 by ocular surface epithelial cells (Fig. 4B) and their increased expression in IL-22 KO mice (Fig. 5D), we hypothesize that IL-22 deficiency promotes Th17 cell generation and infiltration in DED mice. In fact, a previous report has suggested that IL-22 suppresses the infiltration of Th17 cells in intestinal mucosa and maintains mucosal barrier function.[25] Furthermore, our data shows that IL-22 mediated suppression of pathogenic IL17-secreting cells results in reduced DED, particularly in the early induction phase, as demonstrated by high clinical corneal erosion scores in IL-22 KO mice. However, similar high corneal erosion scores in the later stage of disease in both IL-22 KO and WT

mice suggests that IL-22 might be inefficient in regulating severe IL-17-mediated ocular inflammation in chronic or later stage of DED. This could also be due to the low expression of IL-22 in severe inflammation similar to our DED severity and IL-22 correlation data (Fig. 1C) in human.

In summary, these findings provide new insights on both cellular source and function of IL-22 in the pathogenesis of DED. Inverse correlation of high levels of IL-22 in human tears, and its inhibitory effect on Th17 mediated inflammation in mice indicate that IL-22 regulates ocular mucosal inflammation in DED. In addition, the demonstration that IL-22 blockade leads to amplification of Th17 immunity and ocular surface damage suggests its potential as a novel treatment approach. Strategies designed to augment IL-22 levels at the ocular surface, and thereby suppressing Th17 functionality, may be a viable therapeutic approach for DED.

METHODS

Patient enrollment and determination of dry eye disease

The study was approved by the Institutional Review Board and followed the tenets of the Declaration of Helsinki, and informed consents were obtained from all patients. A total of 31 non-Sjögren-type DED patients (11 male, 20 female) with a mean age of 59.3 ± 13.1 years (range: 35 – 78 years) completed the study. The inclusion criteria were as follows: one or more DED-related symptoms, including tightness, foreign body sensation, irritation, red-eye, itching sensation, blurring, or pain; Schirmer's test (without anesthesia) of 5 mm in 5 minutes or less in both eyes; tear break-up time (TBUT) of 5 seconds or less in both eyes; typical DED pattern of superficial punctuate erosion of conjunctiva or cornea. The control group was composed of 18 healthy, age- and sex-matched subjects (6 male, 12 female) who had no history of ocular or systemic disease. The mean age of controls was 57.4 ± 14.1 years (range: 39 – 71 years).

For baseline evaluations, the TBUT, Schirmer's test, fluorescein staining using biomicroscopy, tear sampling, and intraocular pressure were assessed on both eyes of DED patients and healthy controls. We also obtained symptom severity scores from DED patients using the OSDI score. During the data collection period, all evaluations were performed in a blinded fashion. DED parameters and tear sampling were performed by H.S.H. and Y.W.J., blinded to the patient's disease status, and collected tears and prepared them for analysis. The data were then analyzed by E.J.C. who was also blinded to the patient's disease status.

Tear collection and measurements of IL-22 and IL-17 levels

To measure the concentration of IL-22 and IL-17 in patients' tears, a bonded 2.0 × 10 mm polyester fiber rods was used to collect total TF from conjunctival sac, as previously reported.[37, 38] Briefly, a polyester wick was applied to tear meniscus of lower conjunctival sac and collected samples were stored at −70°C for mass spectrophotometric analysis. In addition to the TF, which is a mixed fluid of the secretion from the LGs, meibomian glands, and corneoconjunctival cells, pure LF was collected. The detailed methods for collecting LF are shown in Supplement 1 (Supplementary Video file).

All Nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments were carried out on an Applied Biosystem/MDS Sciex 4000 Qtrap quadrupole mass spectrometer (AB/MDS Sciex, Concord, Canada) that was equipped with a turbo-ion spray source. The mass spectrometer was coupled with an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA). Data were acquired and processed using Analyst software (version 1.4.2, AB/MDS Sciex). The chromatographic separation was achieved with a Synergi Hydro-RP (C18) 50 × 2.1 mm I.D., 4 μm 80-Å particles column (Phenomenex, Torrance, CA) at 40 °C. The mobile phase was a binary gradient using 40 % acetonitrile as solution A and 100 % methanol as solution B. The flow rate was 0.6 ml/min. In addition to the IL-22 and IL-17, total protein concentrations in LF (Control: 6.2 ± 3.6 mg/ml; DED: 4.1 ± 3.8 mg/ml, $p = 0.033$ by Mann-Whitney U test) and TF (Control: 7.9 ± 3.2 mg/ml; DED: 13.9 ± 5.2 mg/ml, $p = 0.029$ by Mann-Whitney U test) was also estimated using LC-MS/MS.

Mouse model of dry eye disease

Six- to 8-week-old male C57BL/6 mice (Charles River Laboratory, Wilmington, MA) and IL-22 KO mice (UC Davis MMRRC, Davis, CA) were used in accordance with the standards of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by the Yonsei University College of Medicine. The *in vivo* mouse experiments were performed at the Institute of Vision Research at the Yonsei University College of Medicine. DED was induced in mice as described previously.[6, 7, 39, 40] In brief, mice were exposed to a controlled environment chamber (CEC), which allows the controlled regulation and maintenance of the temperature (21 – 23 °C), relative humidity (< 30 %), and airflow (15 L/min). Clinical signs such as corneal erosions were assessed by corneal fluorescein staining using 1% fluorescein (Sigma-Aldrich, St. Louis, MO) according to the standard National Eye Institute scoring system. One week post DED induction, mice were euthanized and tissues were collected for immunohistochemistry and molecular studies. For *in vivo* IL-22 blockade experiments, 100 μg/mouse of αIL-22 (AF582, R&D Systems, Minneapolis, MN) or PBS were intravenously injected into WT mouse at 2-hour before placing them into the CEC and then daily for 3 days.

Bone marrow chimera experiments

WT B6 and IL-22 KO mice between 6- and 8-week of age were used to make BM chimera. At the day of BM transfer, mice were given lethal total body irradiation (950 – 1000 rad) from a 137 Cs source. Four hours later, they were reconstituted with WT or IL-22 KO mice BM cells (1×10^7) that had been harvested from the femurs of age-matched mice. Experimental transfers were as follows: WT BM cells into irradiated IL-22 KO mice (WT BM → IL-22 KO host) and IL-22 KO BM cells into irradiated WT mice (IL-22 KO BM → WT host). Animals were allowed to reconstitute for 45 days. Before DED induction, only healthy mice with no obvious signs of graft-vs-host disease or other illness were used in experiments. DED induction, sacrifice, and securement of the tissues were performed as described above. At day seven post-DED induction, the cornea and conjunctiva were harvested to analyze the infiltration of Th17 cells using flow cytometry.

Immunohistochemistry

Whole corneas and LGs were fixed in 4 % paraformaldehyde and histological cross sections (7 μm thick) were mounted on poly-L-lysine coated slides. Cross sections were then deparaffinized and stained with anti mouse primary (IL-22, GTX109659, GeneTex, Zeeland, MI; IL-22R1, ab211675, Abcam, Cambridge, UK; IL-10R2, SC-271969, Santa Cruz Biotech., Santa Cruz, CA) overnight at 4 °C. Sections were then incubated with peroxidase-conjugated streptavidin for 20 minutes at room temperature. Protein expression was detected using diaminobenzidine chromogen and 0.05 % H_2O_2 and evaluated under a florescent microscope (BX51-FL, Olympus, Tokyo, Japan). The sections were also counterstained with Meyer's hematoxylin (DAKO, Glostrup, Denmark). Human skin and colon tissue were used as positive controls (data not shown). Sections were observed under a light microscope (Axio Imager 2, Carl Zeiss, Oberkochen, Germany). For, immunofluorescence staining, anti-mouse IL-17 antibody (ab79056, Abcam), Goat anti-rabbit IgG (ab150077, Abcam), and Alexa Fluor 594 conjugated anti mouse CD4 antibody (100446, BioLegend, San Diego, CA) were used. The sections were observed with the confocal microscopy (LSM 800, Carl Zeiss).

Quantitative PCR

Corneas and LGs were harvested from control and DED mice. RNA was isolated using the RNeasy Micro Kit (Qiagen, Valencia, CA) and reversed transcribed into cDNA with Superscript III Kit (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) with pre formulated primers and StepOnePlus Real-Time PCR detection system (Applied Biosystems, Foster City, CA). The results were analyzed by the comparative threshold cycle method. GAPDH was used as an internal control, and data were normalized to untreated controls.

Human corneal epithelial cell culture and hyperosmotic stimulation

Human corneal limbal tissue was harvested from donor corneal buttons following keratoplasty, in accordance with the tenets of the Declaration of Helsinki and with the permission of the Institutional Review board. Corneal limbal tissues were prepared and cultured as described previously.[41, 42] In brief, cells were cultured for a period of 18 hours in a hyperosmolar serum-free media (380m osmol; achieved by adding sodium chloride) in the presence or absence of murine recombinant IL-22 (BioLegend). The actual osmolality of medium was confirmed with an ohmmeter (Model 3320, Advanced instrument, Norwood, MA).

Flow cytometry

Single-cell suspensions of harvested corneas were prepared by collagenase digestion and blocked with an anti-FcR monoclonal antibody for 30 minutes at 4 °C in 1 % bovine serum albumin/0.02 % NaN_3/PBS. Isolated cells were stained with fluorochrome conjugated antibodies and appropriate isotype controls (BioLegend) for 45 minutes at 4 °C. For intracellular staining, cells were first stimulated with Phorbol 12-myristate 13-acetate (PMA)/ionomycin (Sigma-Aldrich) for 6 hours in the presence of GolgiStop (BD Biosciences, San Jose, CA), and then fixed and permeabilized using Fix/Perm (eBioscience,

San Diego, CA). Stained cells were analyzed with a FACSAria™ flow cytometer (BD Biosciences).

ELISA

Protein levels of cytokines in mouse LG tissues and in culture supernatant of hCECs were quantified using commercially available ELISA kits: human IL-17 and IL-22 (D1700 and D2200, respectively; R&D Systems), human IL-1β, IL-6, IL-8, IL-23, TNF-α (437005, 430505, 431505, 435407, and 430205, respectively; BioLegend) and mouse IL-6, IL-17, IL-22 and IL23 (431305, 436205, 436305, and 433704, respectively; BioLegend).

Statistical analysis

Statistical analyses were performed using SPSS for Windows (version 21.0; SPSS Inc., Chicago, IL). Testing for the normality of distribution was performed by using the Kolmogorov–Smirnov test. For *in vivo* and *in vitro* data that was normally distributed, the independent t-test was performed to compare the differences between the two groups and one-way ANOVA test with Bonferroni correction was used to make comparisons among three or more groups. A linear mixed model with Bonferroni correction for repeated measures covariance pattern with unstructured covariance within samples was used for estimating significant differences between groups over time. As the majority of the clinical data including tear cytokine levels did not show a normal distribution, non-parametric tests were adopted. Analyses included the frequency for categorical data and the median with IQR for continuous data. The Mann-Whitney U test was performed to compare the differences between the two groups and the Kruskal-Wallis test with Bonferroni-Dunn's procedure was used to compare the groups for continuous variables. Correlation between tear cytokine levels and dry eye symptoms was assessed using Spearman's correlation test. A p value of < 0.05 was considered significant different. Results are presented as mean ± standard errors of means (s.e.m).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Barabino S, Chen Y, Chauhan S, Dana R. Ocular surface immunity: homeostatic mechanisms and their disruption in dry eye disease. Prog Retin Eye Res. 2012; 31(3):271–285. [PubMed: 22426080]

2. Chauhan SK, Dana R. Role of Th17 cells in the immunopathogenesis of dry eye disease. Mucosal Immunol. 2009; 2(4):375–376. [PubMed: 19532120]

3. Johnson ME, Murphy PJ. Changes in the tear film and ocular surface from dry eye syndrome. Prog Retin Eye Res. 2004; 23(4):449–474. [PubMed: 15219877]

4. Niederkorn JY, Stern ME, Pflugfelder SC, De Paiva CS, Corrales RM, Gao J, et al. Desiccating stress induces T-cell mediated Sjogren's Syndrome-like lacrimal keratoconjunctivitis. J Immunol. 2006; 176(7):3950–3957. [PubMed: 16547229]

5. De Paiva CS, Chotikavanich S, Pangelinan SB, Pitcher JD 3rd, Fang B, Zheng X, et al. IL-17 disrupts corneal barrier following desiccating stress. Mucosal Immunol. 2009; 2(3):243–253. [PubMed: 19242409]

6. Chauhan SK, El Annan J, Ecoiffier T, Goyal S, Zhang Q, Saban DR, et al. Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. J Immunol. 2009; 182(3):1247–1252. [PubMed: 19155469]

7. Chen Y, Chauhan SK, Lee HS, Saban DR, Dana R. Chronic dry eye disease is principally mediated by effector memory Th17 cells. Mucosal Immunol. 2014; 7(1):38–45. [PubMed: 23571503]

8. Chauhan SK, Jin Y, Goyal S, Lee HS, Fuchsluger TA, Lee HK, et al. A novel pro-lymphangiogenic function for Th17/IL 17. Blood. 2011; 118(17):4630–4634. [PubMed: 21908425]

9. Dohlman TH, Chauhan SK, Kodati S, Hua J, Chen Y, Omoto M, et al. The CCR6/CCL20 axis mediates Th17 cell migration to the ocular surface in dry eye disease. Investigative ophthalmology & visual science. 2013; 54(6):4081–4091. [PubMed: 23702781]

10. Zheng X, de Paiva CS, Li DQ, Farley WJ, Pflugfelder SC. Desiccating stress promotion of Th17 differentiation by ocular surface tissues through a dendritic cell-mediated pathway. Investigative ophthalmology & visual science. 2010; 51(6):3083–3091. [PubMed: 20130281]

11. Tan X, Sun S, Liu Y, Zhu T, Wang K, Ren T, et al. Analysis of Th17-associated cytokines in tears of patients with dry eye syndrome. Eye (Lond). 2014; 28(5):608–613. [PubMed: 24603428]

12. Song XJ, Li DQ, Farley W, Luo LH, Heuckeroth RO, Milbrandt J, et al. Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. Investigative ophthalmology & visual science. 2003; 44(10):4223–4229. [PubMed: 14507865]

13. Lam H, Bleiden L, de Paiva CS, Farley W, Stern ME, Pflugfelder SC. Tear cytokine profiles in dysfunctional tear syndrome. Am J Ophthalmol. 2009; 147(2):198–205 e191. [PubMed: 18992869]

14. Ji YW, Byun YJ, Choi W, Jeong E, Kim JS, Noh H, et al. Neutralization of ocular surface TNF-alpha reduces ocular surface and lacrimal gland inflammation induced by in vivo dry eye. Investigative ophthalmology & visual science. 2013; 54(12):7557–7566. [PubMed: 24052636]

15. Rubino SJ, Geddes K, Girardin SE. Innate IL-17 and IL-22 responses to enteric bacterial pathogens. Trends Immunol. 2012; 33(3):112–118. [PubMed: 22342740]

16. Yang X, Zheng SG. Interleukin-22: a likely target for treatment of autoimmune diseases. Autoimmun Rev. 2014; 13(6):615–620. [PubMed: 24418299]

17. Rutz S, Eidenschenk C, Ouyang W. IL-22, not simply a Th17 cytokine. Immunol Rev. 2013; 252(1):116–132. [PubMed: 23405899]

18. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol. 2011; 12(5):383–390. [PubMed: 21502992]

19. Lavoie TN, Stewart CM, Berg KM, Li Y, Nguyen CQ. Expression of interleukin-22 in Sjogren's syndrome: significant correlation with disease parameters. Scand J Immunol. 2011; 74(4):377–382. [PubMed: 21645026]

20. Ciccia F, Giardina A, Rizzo A, Guggino G, Cipriani P, Carubbi F, et al. Rituximab modulates the expression of IL-22 in the salivary glands of patients with primary Sjogren's syndrome. Ann Rheum Dis. 2013; 72(5):782–783. [PubMed: 23264342]

21. Pan HF, Li XP, Zheng SG, Ye DQ. Emerging role of interleukin-22 in autoimmune diseases. Cytokine Growth Factor Rev. 2013; 24(1):51–57. [PubMed: 22906768]

22. Ciccia F, Guggino G, Rizzo A, Ferrante A, Raimondo S, Giardina A, et al. Potential involvement of IL-22 and IL-22 producing cells in the inflamed salivary glands of patients with Sjogren's syndrome. Ann Rheum Dis. 2012; 71(2):295–301. [PubMed: 21979002]

23. Li Z, Burns AR, Miller SB, Smith CW. CCL20, gammadelta T cells, and IL-22 in corneal epithelial healing. FASEB J. 2011; 25(8):2659–2668. [PubMed: 21518851]
24. Deng R, Hua X, Li J, Chi W, Zhang Z, Lu F, et al. Oxidative stress markers induced by hyperosmolarity in primary human corneal epithelial cells. PloS one. 2015; 10(5):e0126561. [PubMed: 26024535]
25. Shih VF, Cox J, Kljavin NM, Dengler HS, Reichelt M, Kumar P, et al. Homeostatic IL 23 receptor signaling limits Th17 response through IL-22 mediated containment of commensal microbiota. Proc Natl Acad Sci U S A. 2014; 111(38):13942–13947. [PubMed: 25201978]
26. Qu N, Xu M, Mizoguchi I, Furusawa J, Kaneko K, Watanabe K, et al. Pivotal roles of T-helper 17-related cytokines, IL-17, IL-22, and IL-23, in inflammatory diseases. Clinical & developmental immunology. 2013; 2013:968549. [PubMed: 23956763]
27. Tanaka T, Narazaki M, Kishimoto T. Therapeutic targeting of the interleukin-6 receptor. Annual review of pharmacology and toxicology. 2012; 52:199–219.
28. Waldner MJ, Neurath MF. Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development. Seminars in immunology. 2014; 26(1):75–79. [PubMed: 24447345]
29. Pflugfelder SC, Corrales RM, de Paiva CS. T helper cytokines in dry eye disease. Exp Eye Res. 2013; 117:118–125. [PubMed: 24012834]
30. Kumar P, Subramaniyam G. Molecular underpinnings of Th17 immune-regulation and their implications in autoimmune diabetes. Cytokine. 2014
31. Kang MH, Kim MK, Lee HJ, Lee HI, Wee WR, Lee JH. Interleukin-17 in various ocular surface inflammatory diseases. J Korean Med Sci. 2011; 26(7):938–944. [PubMed: 21738349]
32. Penel Sotirakis K, Simonazzi E, Peguet Navarro J, Rozieres A. Differential capacity of human skin dendritic cells to polarize CD4+ T cells into IL-17, IL-21 and IL-22 producing cells. PloS one. 2012; 7(11):e45680. [PubMed: 23226194]
33. Guo X, Qiu J, Tu T, Yang X, Deng L, Anders RA, et al. Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection. Immunity. 2014; 40(1):25–39. [PubMed: 24412612]
34. Korn LL, Thomas HL, Hubbeling HG, Spencer SP, Sinha R, Simkins HM, et al. Conventional CD4+ T cells regulate IL-22-producing intestinal innate lymphoid cells. Mucosal Immunol. 2014; 7(5):1045–1057. [PubMed: 24448096]
35. Sabat R, Ouyang W, Wolk K. Therapeutic opportunities of the IL-22-IL-22R1 system. Nat Rev Drug Discov. 2014; 13(1):21–38. [PubMed: 24378801]
36. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. Immunity. 2004; 21(2):241–254. [PubMed: 15308104]
37. Lee HK, Ryu IH, Seo KY, Hong S, Kim HC, Kim EK. Topical 0.1% prednisolone lowers nerve growth factor expression in keratoconjunctivitis sicca patients. Ophthalmology. 2006; 113(2):198–205. [PubMed: 16360211]
38. Shim J, Park C, Lee HS, Park MS, Lim HT, Chauhan S, et al. Change in prostaglandin expression levels and synthesizing activities in dry eye disease. Ophthalmology. 2012; 119(11):2211–2219. [PubMed: 22858125]
39. Seo Y, Ji YW, Lee SM, Shim J, Noh H, Yeo A, et al. Activation of HIF-1alpha (hypoxia inducible factor-1alpha) prevents dry eye-induced acinar cell death in the lacrimal gland. Cell Death Dis. 2014; 5:e1309. [PubMed: 24967971]
40. Ji YW, Seo Y, Choi W, Yeo A, Noh H, Kim EK, et al. Dry eye-induced CCR7+CD11b+ cell lymph node homing is induced by COX-2 activities. Investigative ophthalmology & visual science. 2014; 55(10):6829–6838. [PubMed: 25257053]
41. Lee JH, Kim M, Im YS, Choi W, Byeon SH, Lee HK. NFAT5 induction and its role in hyperosmolar stressed human limbal epithelial cells. Investigative ophthalmology & visual science. 2008; 49(5):1827–1835. [PubMed: 18436816]
42. Lee JH, Kim JW, Im YS, Seong GJ, Lee HK. Cyclosporine A induces nerve growth factor expression via activation of MAPK p38 and NFAT5. Cornea. 2011; 30(Suppl 1):S19–24. [PubMed: 21912224]

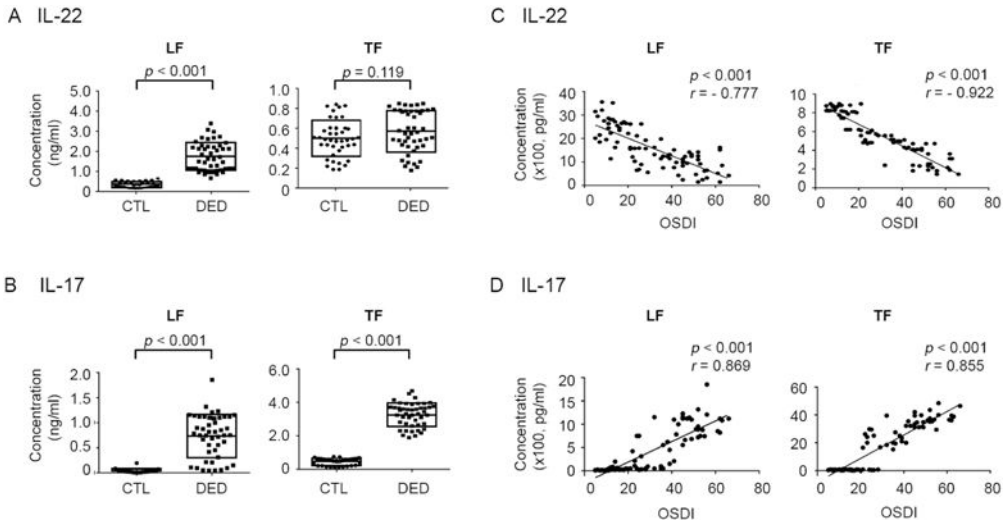

Figure 1.

IL-22 and IL-17 levels in tears of patients with dry eye disease and healthy controls. Lacrimal fluid (LF) from lacrimal glands and total tear fluids (TF) from conjunctival sac were collected using polyester wick from dry eye disease (DED) patients (n=31) and healthy controls (CTL) (n=18). IL-22 and IL-17 levels in LF and TF were estimated using Nano-liquid chromatography tandem mass spectrometry. **A.** IL-22 levels in LF and TF of healthy control and DED patients. **B.** IL-17 levels in LF and TF of healthy controls and DED patients. The box plots bind the interquartile range divided by the median and dots indicate individual values (*p* value by Mann-Whitney U test). **C.** Correlation analysis between ocular surface disease index (OSDI) and IL-22 levels in LF and TF of DED patients. **D.** Correlation analysis between OSDI and IL-17 levels in LF and TF of DED patients. Individual points and lines represent the observed values and regression lines (*r*, Spearman correlation coefficient, $-1 \leq r \leq 1$; *r* and *p* value by Spearman's correlation test).

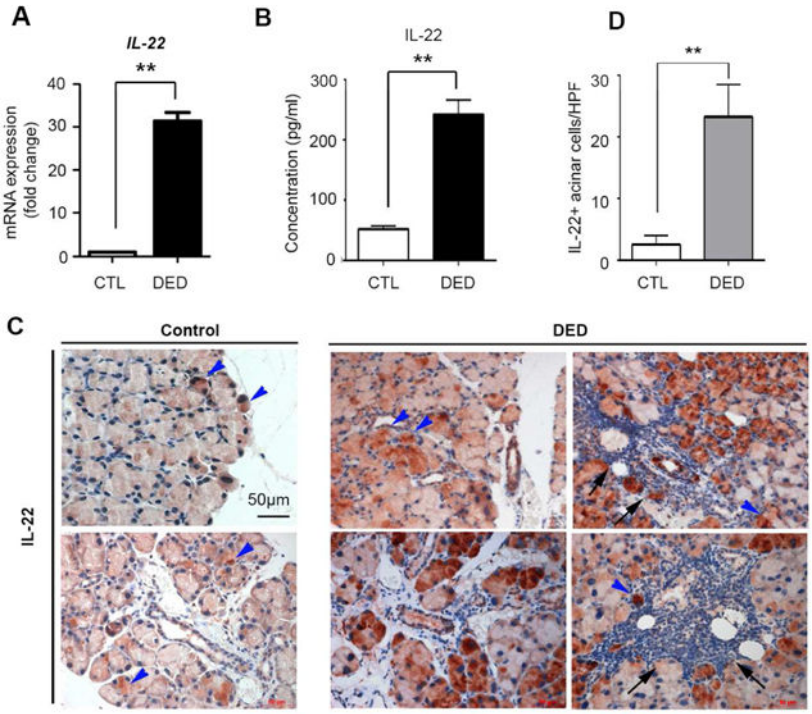

Figure 2.

Expression of IL-22 in the lacrimal glands of mice. Lacrimal glands (LGs) from control (CTL) and dry eye disease (DED) mice were harvested. **A**. IL-22-mRNA expression in LG lysates was quantitated using real-time PCR. **B.** IL-22 protein concentrations in LG lysates was measured by ELISA. **C.** Representative immunohistochemistry micrographs of LG cross sections from two healthy and four DED mice, which were stained for IL-22 (blue arrowhead). Black arrows indicate infiltrated immune cells. D. Bar chart showing quantitation of IL22 producing cells as IL22+ cell /High Power Field (HPF). Data shown are means (bars) ± s.e.m (error bars) of three independent experiments (n = 5 mice/group; **, $p < 0.001$ by independent *t*-test).

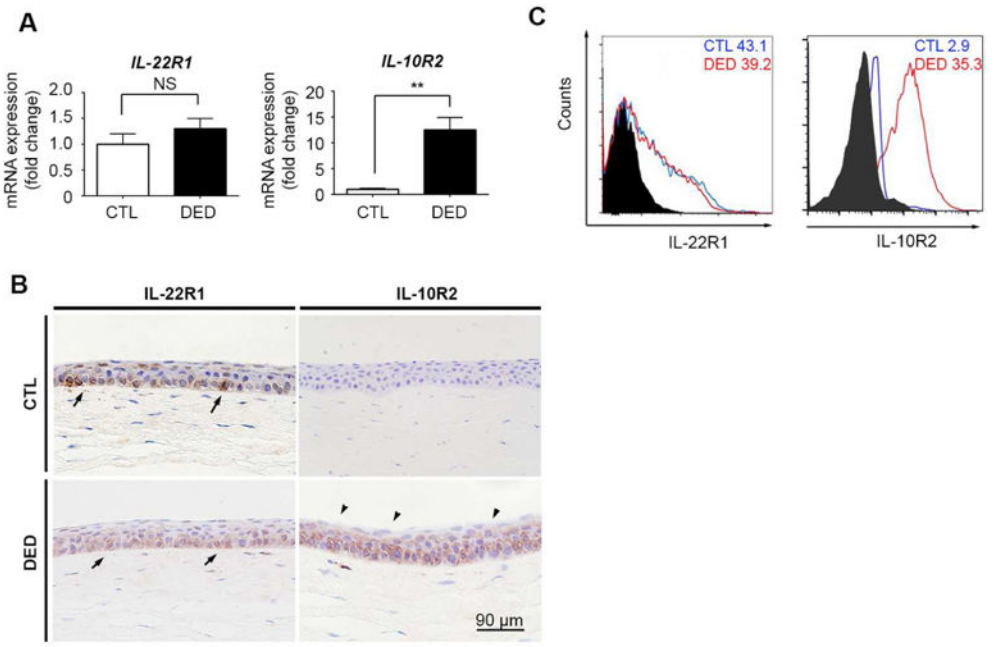

Figure 3.

Expression of IL-22 receptors on the ocular surface of mice. Corneas and conjunctivae from healthy (CTL) and dry eye disease (DED) mice were harvested 7 days after DED induction. **A.** IL-22R1 and IL-10R2 transcripts levels were determined using real-time PCR. **B.** Immunohistochemistry micrographs of corneal cross sections showing expression of IL-22R1 (black arrows) and IL-10R2 (arrowheads) by corneal epithelial cells. **C.** Representative flow cytometry analysis of corneal and conjunctival single cell suspension showing expression of IL-22R1 and IL-10R2. The values shown are means (bars) ± s.e.m (error bars) of three independent experiments (n = 5 mice/group; **, $p < 0.001$ by independent *t*-test; NS, not significant).

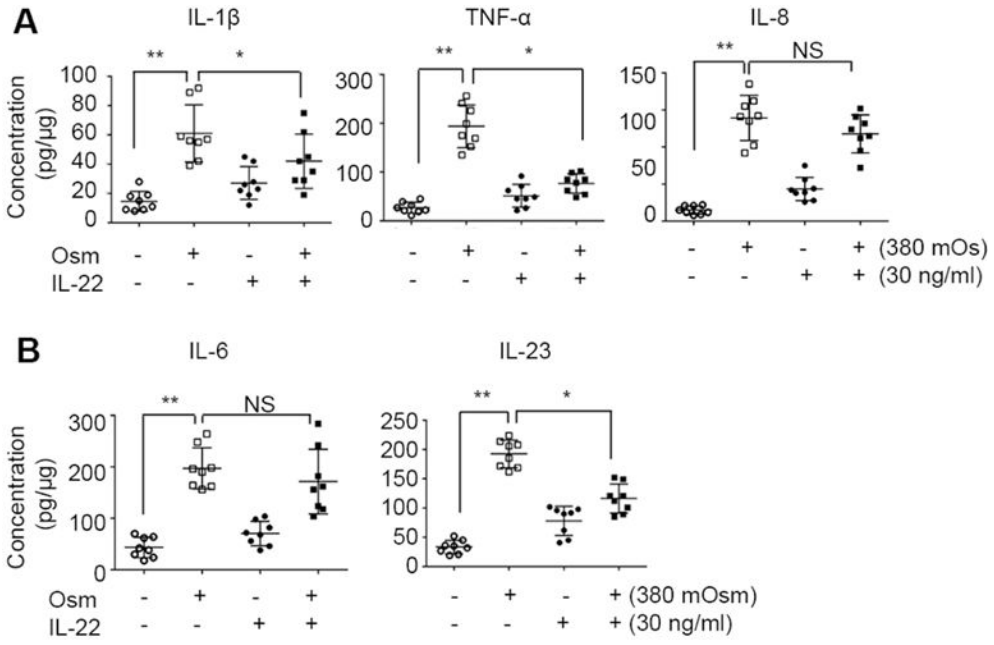

Figure 4.

Effect of IL-22 on human corneal epithelial cell response to desiccating hyperosmolar stress. Human corneal epithelial cells (hCECs) were cultured in 380m osmolar medium for 18 hours in the presence and absence of IL-22 (30 ng/ml). Culture supernatants were collected and ELISA was performed on the inflammatory cytokines IL-1β, TNF α, and IL-8 (**A**), as well as the Th17 inducer cytokines IL-6 and IL-23 (**B**). Results are expressed as means ± s.e.m (error bars) of two independent experiments (n = 8). Dots indicate individual values (*, $p < 0.01$ and **, $p < 0.001$ by one way ANOVA test with Bonferroni correction; NS, not significant)

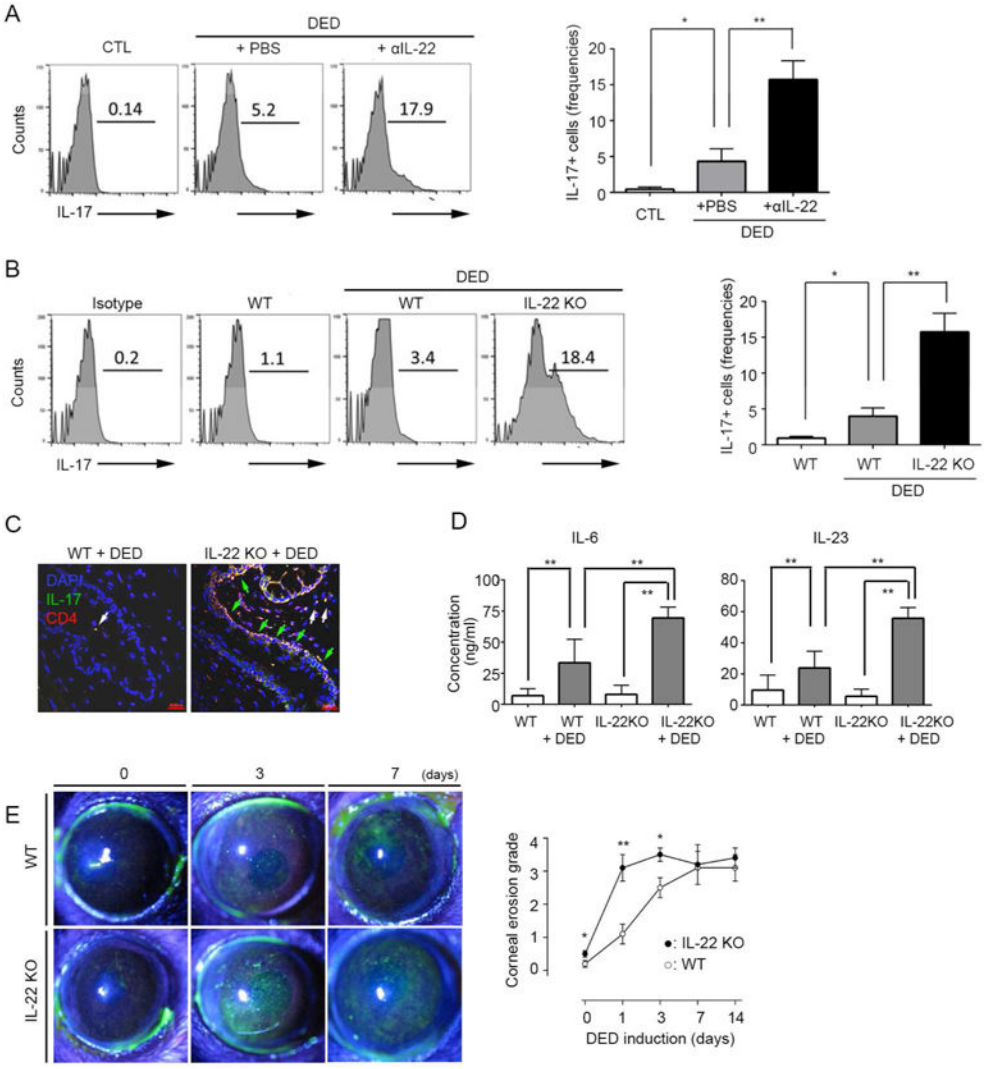

Figure 5.

Effect of IL-22 deficiency on IL-17+ cell infiltration and epitheliopathy in mice with dry eye disease. **A.** IL-22 neutralizing antibodies or PBS were intraperitoneally injected into wild type (WT) mice with dry eye disease (DED) and analyzed on day 7. Naïve mice served as healthy controls (CTL). Single cell suspension of cornea and conjunctiva were stained with anti-IL-17 antibodies and flow cytometry was performed. **B.** Corneas and conjunctivae were harvested from WT and IL-22 knockout (KO) mice with DED, and IL-17+ cell frequencies (%) were analyzed using flow cytometry. The data shown are means (bars) ± s.e.m (error bars) of three independent experiments (n = 5 mice/group; *, $p < 0.01$ and **, $p < 0.001$ by one way ANOVA test with Bonferroni correction). **C**. Representative confocal micrographs of conjunctiva showing co-localization of CD4+ (red) and IL-17+ (green) cells (white arrow) and infiltration of these cells into conjunctival epithelia (green arrows) at seven-day post DED induction. **D.** ELISA assay for IL-6 and IL-23 concentration from corneoconjunctival tissue at 7 days after DED induction. The results shown are means (bars) ± s.e.m (error bars) of three independent experiments (n = 4 mice/group; **, $p < 0.001$ by by one way ANOVA test with Bonferroni correction). **E**. Representative images of fluorescein stained ocular

surface and corneal erosion grade of WT (empty circle) and IL-22 KO (filled circle) after DED induction. Results are expressed as means (individual points) ± s.e.m (error bars) of three independent experiments (n = 4 mice/group; *, $p < 0.01$ and **, $p < 0.001$ by a linear mixed model with Bonferroni correction).

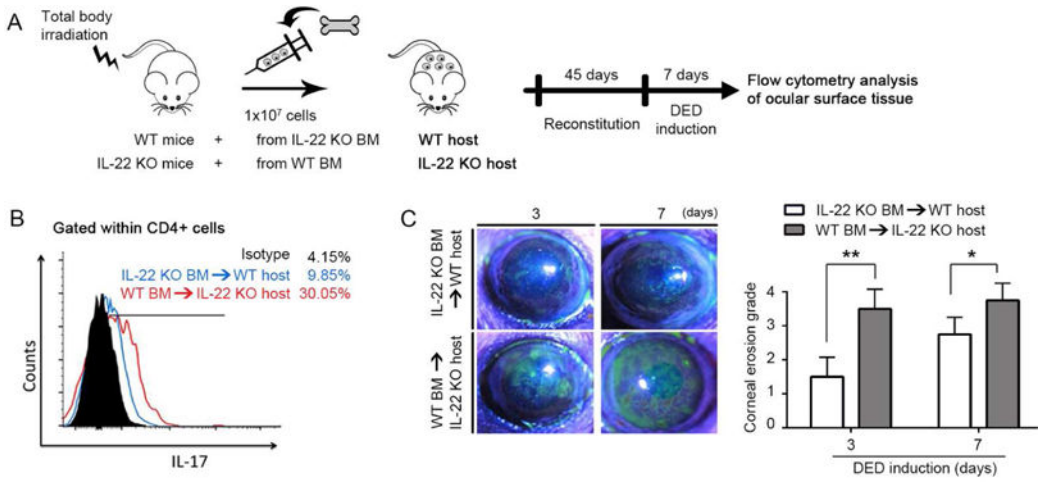

Figure 6.

Non-immune cell-derived IL-22 prevents ocular surface infiltration of pathogenic Th17 cells during dry eye disease. **A**. Schematic illustration of bone marrow (BM) chimera experiments. **B**. Representative histograms showing the frequencies of IL-17 secreting cell (Gated within CD4+ cell) at the ocular surface of wild type (WT) host with IL-22 knockout (KO) BM (Blue) and IL-22 KO host with WT BM (Red) after dry eye disease (DED) induction. **C**. Representative images of fluorescein stained ocular surface of BM chimeras after DED induction. The values shown are means (bars) ± s.e.m (error bars) of two independent experiments (n = 4 mice/group; *, $p < 0.01$ and **, $p < 0.001$ by independent t-test).