Correlations between Tear Cytokines, Chemokines, and Soluble Receptors and Clinical Severity of Dry Eye Disease

Kyung-Sun Na,[1–4] *Jee-Won Mok,*[2,4] *Ja Yeon Kim,*[2] *Chang Rhe Rho,*[2,3] *and Choun-Ki Joo*[2,3]

Purpose. To determine cytokine and chemokine concentrations in the tears of patients with dry eye disease (DED) and analyze the possible relationships with the clinical severity of DED.

Methods. Patients were examined using the Ocular Surface Disease Index, corneal and conjunctival staining, tear breakup time, and impression cytology. They were divided into four groups according to the Dry Eye Workshop severity classification. Tears were collected from 133 patients with DED and 70 healthy controls. Concentrations of cytokines, chemokines, and soluble receptors in collected tear samples were analyzed using current technology with a Human Cytokine/Chemokine kit, a Human Cytokine/Chemokine Panel, and a Human Soluble Cytokine Receptor Panel.

Results. The levels of cytokines interleukin (IL)-1β ($P < 0.05$), IL-6 ($P < 0.001$), IL-16 ($P < 0.001$), IL-33 ($P < 0.05$), G-CSF ($P < 0.001$), and transforming growth factor (TGF)-α ($P < 0.05$) were significantly higher in patients with DED, whereas those of cytokines IL-4 ($P < 0.001$), IL-12 (p40) ($P < 0.001$), IL-17A ($P < 0.05$), and interferon-γ ($P < 0.001$) were significantly lower. The levels of Fractalkine (chemokine [C–X3–C motif] ligand 1; CX3CL1), MCP-1 (chemokine [C–C motif] ligand 2; CCL2), MIP-1δ (chemokine [C–C motif] ligand 15; CCL15), and ENA-78 (chemokine [C–X–C motif] ligand 5; CXCL5) ($P < 0.001$, respectively) and soluble receptors, sIL-1RI ($P < 0.05$), soluble glycoprotein (sgp) 130 ($P < 0.05$), sIL-6R ($P < 0.001$), soluble epidermal growth factor receptor ($P < 0.05$), and soluble tumor necrosis factor receptor 2 ($P < 0.001$), were higher in patients with DED. There were significant correlations between these molecules and the clinical severity of DED.

Conclusions. Fifteen molecules were elevated in the tears of patients with DED; four molecules were decreased. Although the levels of sIL-6R, sIL-6R, and sgp130 may be potential indicators of the homeostatic process, an increase in the levels of IL-6 and IL-1 β are the earliest observable changes in patients with DED. Further study on the biomarkers in the pathogenesis of DED and treatment target modalities would be needed. (*Invest Ophthalmol Vis Sci.* 2012;53:5443–5450) DOI: 10.1167/iovs.11-9417

Dry eye disease (DED) is a highly prevalent inflammatory disease of the lacrimal functional unit caused by multifactorial reasons. It can lead to a chronic ocular surface disease and impaired visual function, thus reducing quality of life.[1–3] Although there are different pathogenic mechanisms responsible for DED, increasing evidence suggests that all forms of DED are characterized by varying ocular surface inflammation.[4] The inflammation is sustained by ongoing activation and infiltration of pathogenic immune cells, primarily $CD4^+$ helper T cells in the conjunctiva and $CD11b^+$ monocytic cells in the cornea.[5]

Increased production and activation of proinflammatory cytokines and proteolytic enzymes by the affected cornea, conjunctiva, and glandular epithelial cells, as well as the inflammatory cells that infiltrate the tissue, were reported in detail previously.[6–8] Increased concentrations of proinflammatory cytokines and chemokines in tears, such as interleukin 1 (IL-1), Il-6, IL-8, and tumor necrosis factor-α (TNF-α), have been investigated.[8–15] Furthermore, some studies suggest that increased levels of several inflammatory cytokines are correlated with clinical parameters in DED.[9,12–15] The studies found that cytokine and chemokine levels are significantly correlated with various clinical diagnostic tools such as the Schirmer test, tear breakup time (tBUT), tear clearance rate, and corneal and conjunctival staining. However, whether the inflammation is a cause or consequence of DED is still veiled, and which cytokines or chemokines play major roles in the pathogenesis of DED remain unclear. It is essential to evaluate biological markers and diagnostic applications accurately to establish treatment strategies to encompass the multifactorial nature of DED.

The diagnosis of DED, especially in the early stage, has been hampered by a lack of objective tests with sufficient and specific clinical tools. Although the symptoms of DED are common, there is no correlation between symptoms and signs, particularly in mild DED.[16] Moreover, there is a lack of data on the clinical usefulness of objective tests in the diagnosis of DED.[1]

We hypothesize that the levels of some cytokines, chemokines, and soluble receptors in tear fluid play physiologic and some pathogenic roles on the ocular surface. In addition, determining which molecules are correlated with DED severity will result in a better understanding of the pathogenesis of DED, which molecules can be used as biomarkers in the early stage of disease, and ultimately, what topical modulator can be used as a treatment modality to inhibit the disease pathogenesis selectively. To test this hypothesis, we investigated the levels of a wide panel of cytokines, chemokines, and soluble receptors in tear samples from a large number of patients and healthy controls using a multianalyte profiling bead-based assay.

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Table 1. Cytokines, Chemokines, and Soluble Receptors for Tear Quantification of Patients with Dry Eye Disease

Human Cytokine Human Cytokine/Chemokine Panel I	EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN α2, IFN γ, IL-1ra, IL-1α, IL-1β, IL-2, sIL-2Rα, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, sCD40L, TGFβ, TNFα, TNFβ, VEGF
Human Cytokine Human Cytokine/Chemokine Panel II	MCP-2, MCP-4, ENA-78(CXCL5), SDF-1α+β (CXCL12), BCA-1 (CXCL13), I-309 (CCL1), MIP-1δ /MIP-5 (CCL15), TARC (CCL17), 6Ckine (CCL21), EOTAXIN-2 (CCL24), EOTAXIN-3 (CCL26), CTACK (CCL27), IL-23, LIF, TPO, TRAIL (TNFSF10), SCF, TSLP, IL-20, IL-IL-28, IL-16, IL-33 (NF-HEV)
Human Soluble Cytokine Receptor	Soluble CD30 (sCD30, sTNFRSF8), soluble Epidermal Growth Factor Receptor (sEGFR), soluble gp130 (sgp130), soluble Interleukin-1 Receptor Type I (sIL-1RI, sCD121a), soluble Interleukin-1 Receptor Type II (sIL-1RII, sCD121b), soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR1, sFlt-1), soluble Vascular (CD124), soluble Interleukin-6 Receptor (sIL-6R, CD126), soluble Receptor for Advanced Interleukin-2 Receptor alpha (sIL-2Rα, CD25), soluble Interleukin-4 Receptor (sIL-4R), Glycation Endproducts (sRAGE), soluble Tumor Necrosis Factor Receptor I (sTNFRI, TNFRSF1A), soluble Tumor Necrosis Factor Receptor II (sTNFRII, TNFRSF1B), soluble Vascular Endothelial Growth Factor Receptor 2 (sVEGFR2, sFlk-1, sKDR), and soluble Vascular Endothelial Growth Factor Receptor 3 (sVEGFR3, sFlt-4)

Methods

Study Subjects

The study population comprised 133 patients with DED and 70 healthy controls. Written informed consent was obtained from all subjects, and the Medical Ethics Committee of the Catholic University of Korea approved the study. The diagnosis of DED was based on the assessment of signs and symptoms by three observers (K.S. Na, C.R. Rho, and C.K. Joo) and according to each patient's report of symptoms of ocular irritation as assessed by the Ocular Surface Disease Index (OSDI) score, Schirmer test (without anesthesia), tear film breakup time (tBUT), corneal staining with fluorescein, and conjunctival staining with lissamine green. These tests were used to qualify patients for inclusion in the study and for grading DED severity.

Subjective symptoms were graded on a numerical scale of 0 to 4 using the OSDI score: 0, none of the time; 1, some of the time; 2, half of the time; 3, most of the time; 4, all of the time. A 5-minute Schirmer test was performed using sterile strips without anesthesia. The Schirmer strip was placed at the notch of the inferior fornix; after 5 minutes, the strip was removed and measured at the point of maximum wetting. The tBUT was measured after placing a sodium fluorescein paper at the lower tarsal conjunctiva. The patients were asked to blink; the time before the defect appeared in the stained tear film was measured and recorded as the tBUT. For corneal fluorescein staining, the entire cornea was examined by slit-lamp evaluation with a yellow barrier filter and cobalt blue illumination. Anterior segment photographs were obtained and the stains were graded using the National Eye Institute method, a standardized scale of 0 to 3 for each of the five regions of the cornea: central, inferior, nasal, superior, and temporal. Conjunctival staining with lissamine green was evaluated according to the Oxford Schema (0–4) for the three regions of the conjunctiva: central, nasal, and temporal.

Patients' signs and symptoms were analyzed for classification into the dry eye severity categories developed from the Dry Eye Workshop (DEWS) report by Asbell et al.[17] Selected criteria including the OSDI, Schirmer test, tBUT, corneal staining, and conjunctival staining were used to classify disease severity. Patient evaluations were rated according to the numeric dry eye severity scale for each diagnostic test. Each patient was assigned an overall dry eye severity grade of 1, 2, 3, or 4 based on the mode and arithmetic mean of the individual severity grades for the selected criteria.

The exclusion criteria were as follows: an inflammatory disease not associated with dry eyes; ocular trauma or surgical history within the previous year; pregnancy; systemic diseases such as diabetes and hypertension; rheumatologic, hematologic, and respiratory diseases; systemic infection; and any other significant disease. During the study, patients were excluded if they were found to fulfill any of the above exclusion criteria, even if they were unaware of fulfilling any of these criteria at the time of enrollment.

Age-matched healthy controls were selected from visitors who underwent regular medical check-ups at the Health Promotion Center of St. Mary's Hospital in Seoul, Korea. The inclusion criteria were as follows: no subjective ocular symptoms; no abnormal signs of the eye after ophthalmic examination by an ophthalmologist (K.S. Na); no ocular trauma or surgical history; no physician-confirmed systemic diseases as based on the results of their medical examinations; and no medication history that could affect ocular surface physiology.

Tear Sample Collection

Polyurethane minisponges were obtained commercially (PeleTim; VOCO GmbH, Cuxhaven, Germany). Specialized nurses carried out the procedures. A single polyurethane minisponge was laid on the outer third of the lower eyelid margin. After 5 minutes of tear collection, the sponge was recovered and placed in the narrow end of a truncated Gilson micropipette tip adapted to a 1.5-mL tube (Eppendorf, Fremont, CA) and centrifuged at 6000 rpm for 5 minutes. Tear samples from both eyes were pooled and immediately stored at –80°C until they were used for the immunoassay.

Multiplex Bead Analysis

Cytokines, chemokines, and soluble receptors were analyzed using a a commercial assay system of immunoassay kits and panels (Millipore MILLIPLEX Human Cytokine Human Cytokine/Chemokine Panel I Premixed 39 Plex [MPXHCYTO60KPMX39], Millipore MILLIPLEX Human Cytokine Human Cytokine/Chemokine Panel II Premixed 23 Plex [MPXHCYP2PMX23], and Millipore MILLIPLEX Human Cytokine Human Soluble Cytokine Receptor Panel, Premixed 14 Plex [HSCR-32K-PMX14]; Millipore, Billerica, MA) using a magnetic bead-based immunoassay kit (Luminex 200; Luminex Corp., Austin, TX). The quantified cytokines, chemokines, and soluble receptors are listed in Table 1. Tear samples were incubated with antibody-coated capture beads overnight at 4°C. Washed beads were further incubated with biotin-labeled anti-human cytokine antibodies, followed by streptavidin–phycoerythrin incubation. The standard curves of known concentrations of recombinant human cytokines/chemokines/soluble receptors were used to convert fluorescence units to concentrations (pg/mL or ng/mL). To calculate molecular concentrations in tear samples, we analyzed the median fluorescent intensity data using a 5-parameter logistic or spline curve-fitting method. Cytokines, chemokines, and soluble receptors for tear quantification are expressed in Table 1.

Statistical Analysis

The concentrations of cytokines, chemokines, and soluble receptors in the two study groups are expressed as mean ± SD and compared using ANOVA with a post hoc test using a commercial statistical package

Table 2. Demographic Characteristics of Patients with Dry Eye Disease

Characteristic	Grade 1		Grade 2		Grade 3	
	OD	OS	OD	OS	OD	OS
Age (y ± SD)	48.9 ± 17.26 (30 to 65)		54.9 ± 13.78 (22 to 88)		53.2 ± 14.23 (17 to 80)	
Sex (Female %)	100		84		60	
OSDI Score (≤12 cutoff; %)	50		32		20	
OSDI Score (13–32 cutoff; %)	30		57		50	
OSDI Score (≥33 cutoff; %)	20		11		30	
Osmolarity (mOsm/L; mean ± SD)	285.9 ± 94.19	284.3 ± 93.07	291.9 ± 36.12	290.3 ± 35.91	284.4 ± 69.36	284.6 ± 76.66
TBUT (>10 s cutoff; %)	0	0	0	0	0	0
TBUT (5 s <<10 s cutoff; %)	90	80	54	49	28	25
TBUT (<5 s cutoff; %)	10	20	42	45	55	58
TBUT (immediate; %)	0	0	4	6	18	18
OD Schirmer (>15 mm; %)	60	30	10	12	3	3
OD Schirmer (14–9 mm; %)	20	40	7	11	8	5
OD Schirmer (8–4 mm; %)	10	30	45	34	35	35
OD Schirmer (<4 mm; %)	10	0	39	43	55	58
Corneal staining (Grade 0; %)	0		0		0	
Corneal staining (Grade 1; %)	80	80	48	48	13	15
Corneal staining (Grade 2; %)	10	10	45	43	55	45
Corneal staining (Grade 3; %)	10	10	7	9	33	40
Corneal staining (Grade 4; %)	0	0	1	1	0	0
Conjunctival staining (Grade 1; %)	100	100	38	38	15	16
Conjunctival staining (Grade 2; %)	0	0	56	54	49	45
Conjunctival staining (Grade 3; %)	0	0	6	7	36	39

(SPSS 14.0 Statistical Package for Windows; SPSS Inc., Chicago, IL). The level of statistical significance was set at $P < 0.05$.

Results

Demographic Data

The demographic data of the patients with DED are presented in Table 2.

Tear Cytokine and Chemokine Concentrations

Of the 65 molecules analyzed, IL-1β, IL-6, Il-16, IL-33, transforming growth factor alpha (TGF-α), granulocyte colony-stimulation factor (G-CSF), fractalkine (CX3CL1), monocyte chemotactic protein-1 (MCP-1; CCL2), macrophage inflammatory protein-1 delta (MIP-1δ; CCL15), and epithelial neutrophil–activating peptide (ENA-78; CXCL5) (Figs. 1, 2) in the DED group were significantly elevated compared with the control group. Among these molecules, the concentrations of IL-1β, IL-16, TGF-α, fractalkine (CX3CL1), MIP-1 δ (CCL15), and ENA-78 (CXCL5) increased gradually according to the clinical severity grade, which suggests that the concentrations of the molecules are significantly correlated with clinical severity. On the other hand, IL-12 (p40), interferon gamma (IFN-γ), IL-17A, and IL-4 in the DED group were decreased significantly compared with the control group (Fig. 3).

Tear-Soluble Cytokine Receptors

Of the 14 soluble cytokine receptors, the DED group had significantly higher levels of soluble IL-1 receptor 1 (sIL-1R1), soluble glycoprotein (sgp)-130, sIL-6R, soluble epidermal growth factor receptor (sEGFR), and soluble tumor necrosis factor receptor (sTNFR) 2 than those of the control group. Among these, sIL-1R, sgp130, sIL-6R, and sTNFR2 increased gradually with increasing clinical severity (Fig. 4).

Gene Expression Analysis of IFN-γ and IL-17A

Results of the quantitative polymerase chain reaction (qPCR) for IFN-γ and IL-17A are described in Fig. 5. Compared with the normal controls, patients with DED did not show a significant increase or decrease in the levels of IFN-γ and IL-17A. On the other hand, Sjögren's patients with DED showed higher levels of IFN-γ and IL-17A. Median IFN-γ transcript levels were 0.57 in controls ($n = 3$), 0.50 in DED severity 1 (S1) ($n = 10$), 0.29 in DED S2 ($n = 10$), 0.39 in DED S3 ($n = 10$), and 0.76 in Sjögren's DED ($n = 10$). The median levels of IL-17A were 0.93 in the controls; 1.23, in DED S1; 0.76, in DED S2; 0.58, in DED S3; and 2.19, in Sjögren's DED (data not shown).

Discussion

Desiccating environmental stress and changes in tear fluid composition accompanying lacrimal gland dysfunction appear to trigger ocular surface inflammation.[18,19] Tear cytokine and chemokine assays provide direct evidence of ocular surface inflammation in this condition. Moreover, elevated levels of tear cytokines and chemokines would be superior indicators of disease severity or biomarkers in the early stage of disease compared with the clinical tests used currently. Furthermore, such cytokines and chemokines might be used as selective targets for treatment modalities in DED.

Lam et al.[13] examined the tears of healthy controls and patients with aqueous-deficient dry eye, evaporative dry eye, and mixed type dry eye using microarrays; they reported that proinflammatory cytokines, including IL-1β, IL-6, IL-8, TNF-α, IFN-γ, cystatin SN, lipocalin 1 (LCN 1), and α-1-antitrypsin are distinctly elevated in the DED group. Cystatin SN and IL-6 exhibited the most distinct differences of all tested proteins when comparing controls with subjects with DED. Li et al.[20] also tested several proinflammatory cytokines such as IL-6, TNF-α, and IFN-γ, and reported similar deviations between the tears of Sjögren's patients with DED and controls. Enriquez-de-Salamanca et al.[21] examined the inflammatory molecules in the tears from

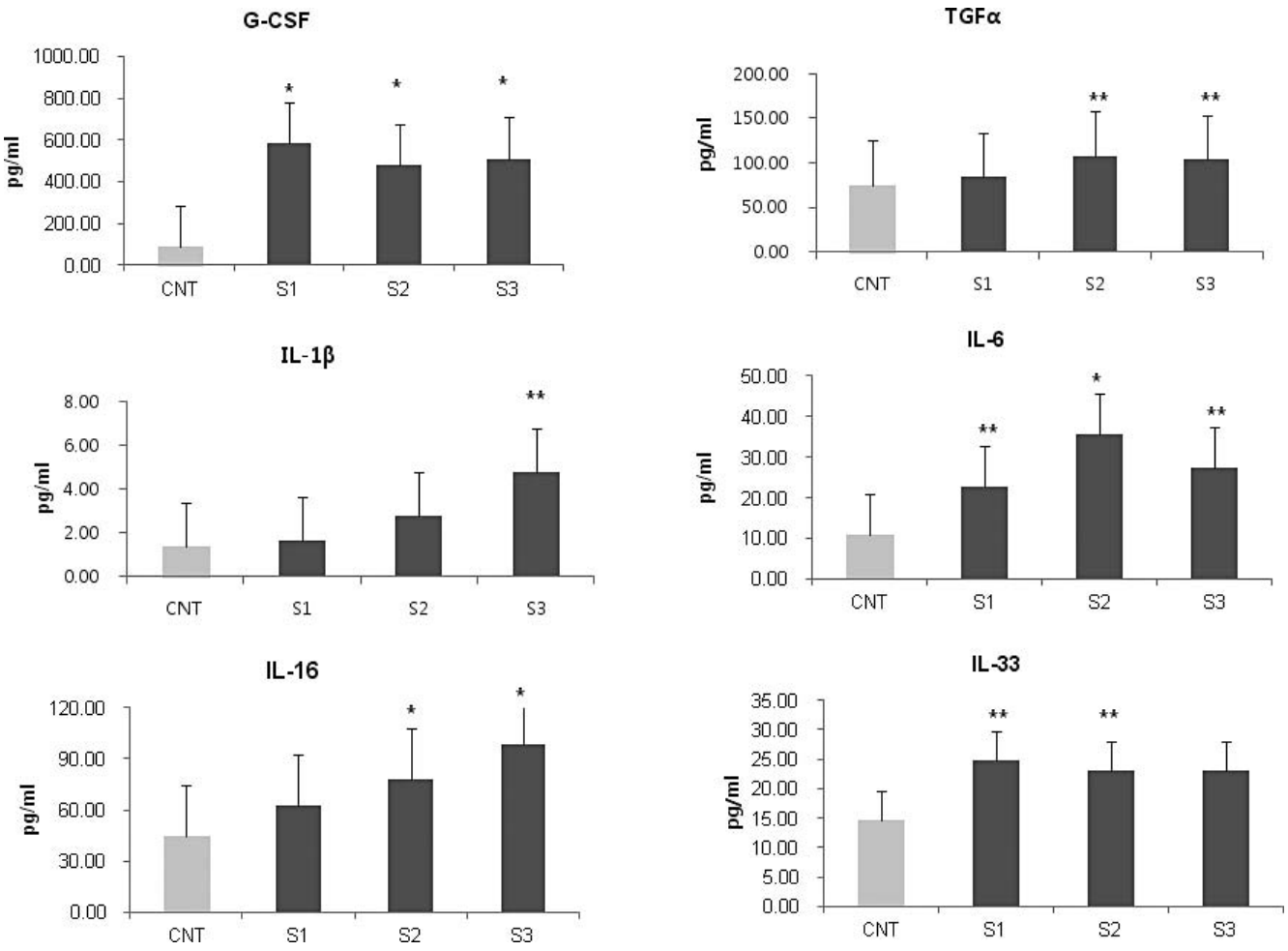

Figure 1. Tear cytokines of the dry eye patients with serial severity and control groups. Significantly elevated levels of IL-1β, IL-6, Il-16, IL-33, TGF-α, and G-CSF in patients with dry eye disease. Control versus patients with dry eye disease; $^{*}P < 0.001$, $^{**}P < 0.05$.

46 eyes of patients with DED and 18 eyes of healthy controls. Both subjective and objective DED studies were performed, and cytokine and chemokine levels were measured by multiplex bead analysis, compared with control levels, and correlated with clinical tests. They reported that pain is correlated with IL-6 and IL-8/CXCL8 levels, and that tBUT is inversely correlated with IL1-Ra. The Schirmer test and tear lysozyme levels were negatively correlated with IL-1Ra, IL-8/CXCL8, fractalkine/CX3CL1, IL-6, and IP-10/CXCL10; conjunctival staining was negatively correlated with EGF and positively with IL-6. Massingale et al.[15] also concluded that tears from patients with dry eye disease contained significantly increased concentrations of cytokines that were also correlated to disease severity.

Our study results show that the levels of IL-1β , IL-6, and their soluble receptors sIL-1R1 and sIL-6R, were significantly elevated in the tears of patients with DED. In addition, IL-33, a member of the IL-1 cytokine family, was also elevated. The IL-1 receptor (IL-1R)/toll-like receptor (TLR) superfamily plays a critical role in the regulation of inflammatory and immune responses.[22] More recently, IL-1 receptor type 1 (IL-R1) signaling was identified as a critical step in the differentiation and commitment of Th17 cells, which mediate the development of autoimmune diseases.[23,24] Upon the binding of IL-1 to IL-1R1, IL-1R accessory protein (AcP) is recruited to form a high-affinity IL-1R1/IL-1RAcP heterodimeric receptor, which initiates the downstream signaling inflammatory cascade. This pathway is strictly regulated by multiple inhibitory molecules, including membrane-bound IL-RII, secreted soluble (s)IL-1R1, sIL-R2, and sIL-1RAcP, the regulatory IL-1R1 antagonist (IL-1R1a), and the IL-1R1 signaling–induced single Ig-IL-1R–related molecule. This negative feedback system suppresses excessive IL-1 signaling and Th17 cell differentiation.[25,26] The simultaneous elevation of IL-1β and sIL-1R1 suggests that natural homeostasis is maintained to inhibit further systemic autoimmune responses following the local inflammation of ocular surfaces; this is strengthened by a gradual decrease in IL-17A in the tears of patients with DED. This is concordant with the results of previous reports in the tears of both aqueous-deficient and evaporative-type patients with DED.[9,21]

IL-6 is reported to be increased in tears and the conjunctival epithelium, and is described as one of the key molecules in DED. In addition, sIL-6R expression in tears is upregulated in chronic inflammatory conditions of the ocular surface.[27,28] Series of IL-6 activities are critical for resolving innate immunity and promoting acquired immunity; this transition is a central event in the resolution of any inflammatory condition, and the disruption of this immunologic switch may potentially distort the immune response, affecting the onset of autoimmune or chronic inflammation.[29,30] A previous study investigated how IL-6 governs the resolution of acute innate immunity and steers the transition to an acquired immune response. IL-6 directs T-cell recruitment by regulating local chemokine secretion (i.e., CXC chemokine ligand 10, CC chemokine ligand 2 [CCL2], CCL4, CCL5, CCL11, and CCL17) and chemokine receptor (CC chemokine receptor 3 [CCR3], CCR4, CCR5, and CXC chemo-

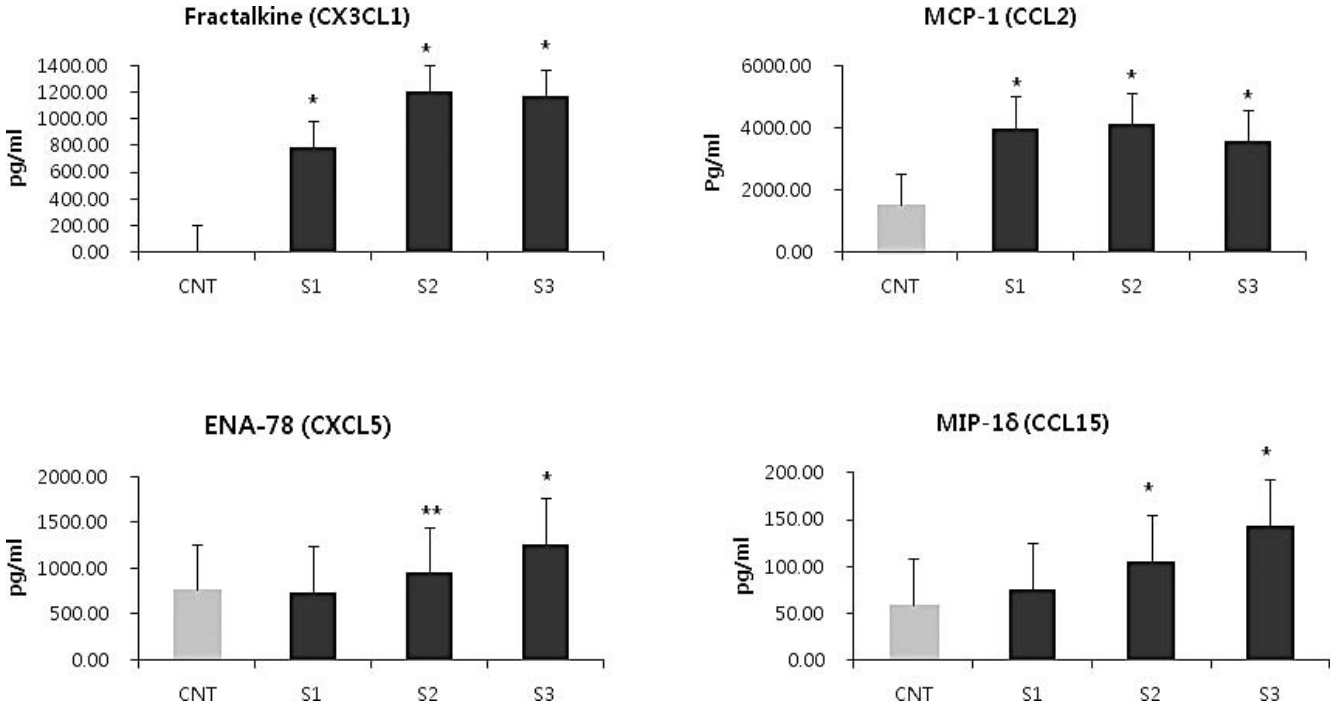

Figure 2. Tear chemokines of the patients with dry eye disease with serial severity and control groups. Significantly elevated levels of CXCR1, CCL2, CCL15, and CCL24 in the patients with dry eye disease. Control versus patients with dry eye disease; $^{*}P < 0.001$, $^{**}P < 0.05$.

kine receptor 3) expression on the $CD3^{+}$ infiltrate.[31] The two models of IL-6 activation are known as classical interleukin activations that occur via membrane-bound IL-6 activation and sIL-6R–mediated signaling (IL6 trans-signaling). In both cases, responses are elicited through engagement with membrane-bound gp130. Moreover, classical IL-6 signaling is unaffected by sgp130, yet preferentially binds the IL-6/sIL-6 complex to antagonize IL-6 trans-signaling. IL-6 trans-signaling is known to contribute to the perpetuation of inflammation; of consequence, high sgp130 might represent an indirect marker of IL-

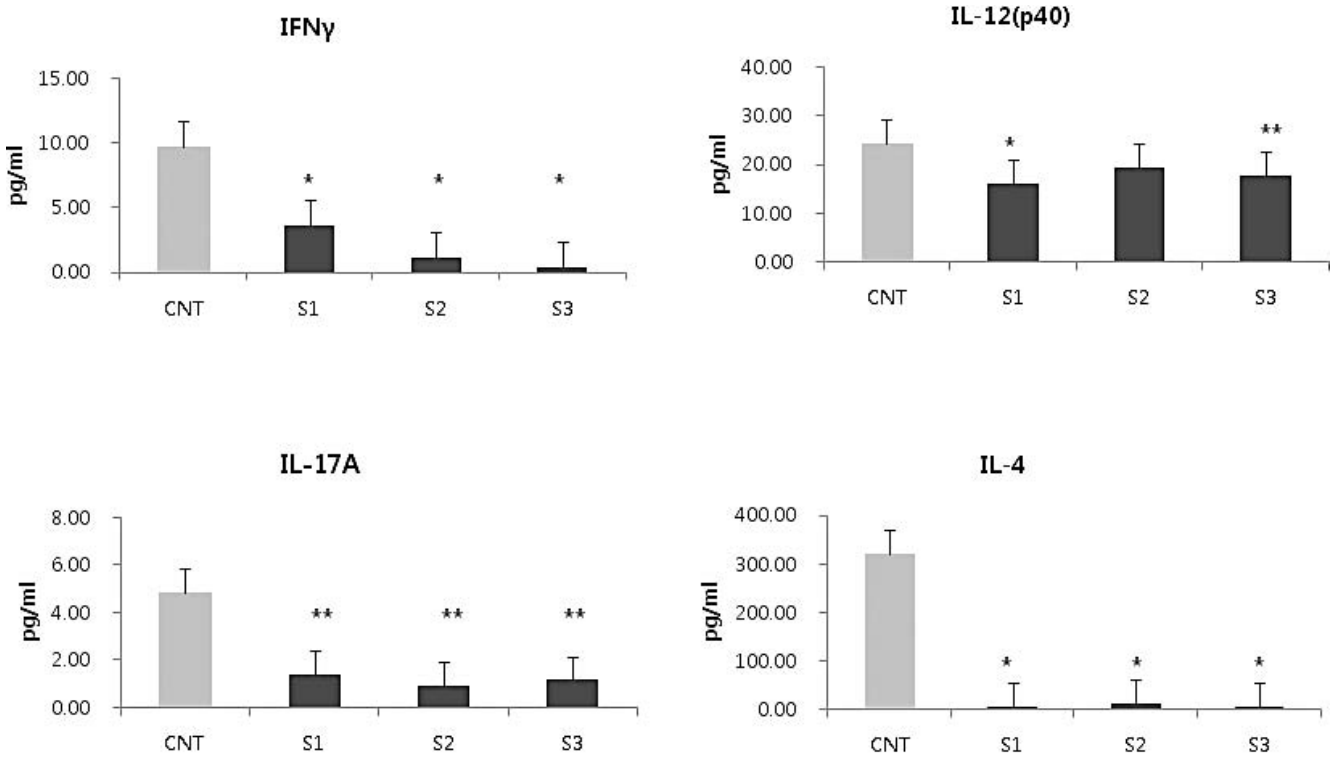

Figure 3. Patients with dry eye disease with serial severity reveal significantly lower levels of the cytokines, IL-12 (p40), IFN-γ, IL-17A, and IL-4 as compared with the group of controls. Control versus patients with dry eye disease; $^{*}P < 0.001$, $^{**}P < 0.05$.

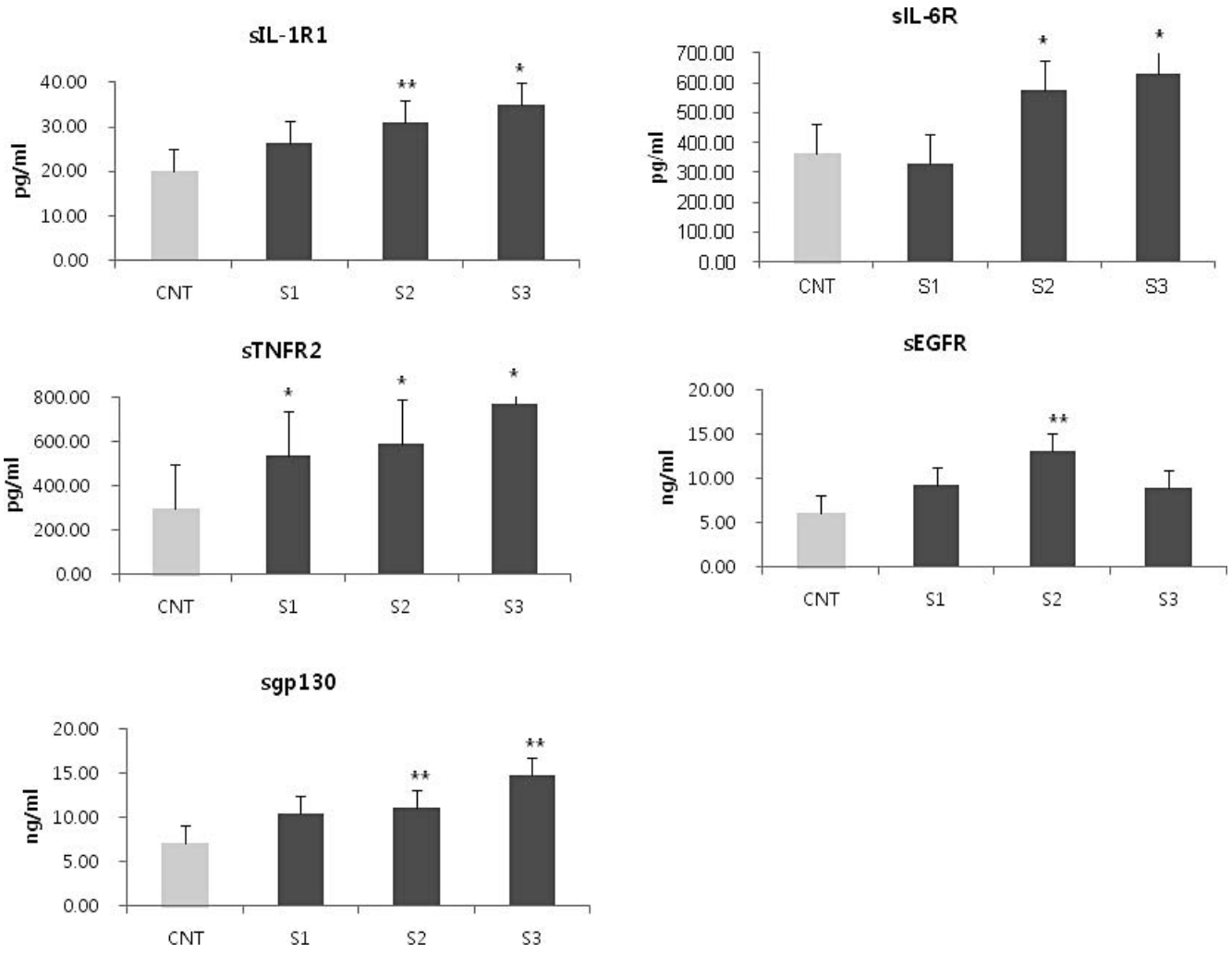

Figure 4. Significantly elevated levels of sIL-1R1, Sgp-130, sIL-6R, sEGFR, and sTNFR 2 in the patients with dry eye disease. Control versus patients with dry eye disease; $^{*}P < 0.001$, $^{**}P < 0.05$.

6–mediated inflammation becoming a chronic process.[32] We assume that the IL-6/sIL-6R complex plays a role in the pathogenesis of DED and that the marked elevation of its natural antagonist, sgp130, may be a result of ocular homeostasis in response to local inflammation.

Innate granulocyte macrophage–colony stimulating factor (GM-CSF) is critical for IL-6 responses by dendritic cells and for the generation of pathologic $CD4^{+}$ T cells producing IL-17 (T helper [Th]17) from naïve T cells.[33,34] A novel proinflammatory subset of Th17, which is distinct from Th1 and Th2, has been suggested to mediate the inflammation associated with several autoimmune diseases.[35,36] The involvement of T cells in the immunopathogenesis of DED is supported by many investigators, including increased T cells in the conjunctiva,[37] the presence of IFN-γ+ T cells in the murine model,[38] and improvement of DED after the administration of a topical T-cell immunosuppressant.[39] The dysfunctions of regulatory T cells (Treg) and pathogenic effector T cells are known to contribute to the pathogenesis of DED in animal models. However, our data showed trace levels of IL-17A and IFN-γ in the tears of patients with DED. To validate the findings, gene expression analysis was performed by qPCR with conjunctival impression cytology samples. The results revealed that unlike Sjögren's DED, which showed higher levels of both IL-17A and IFN-γ than the healthy controls, non-Sjögren's DED showed no significant increase or decrease. There was a tendency of a gradual decrease in the IL-17A levels according to the clinical severity grade; however, these differences were not statistically significant. In terms of the conflicting results with respect to IFN-γ and IL-17A, we assume that the suppressor T-cell population, which is a natural inhibitor of self-reactive Th1 (INF-γ), Th2 (IL-4+), and Th17 (IL-17A) cells,[40] may function to some extent in the mild and early stages of DED. We excluded patients with DED severity 4 because of insufficient tear volume. If patients with severe and late-stage DED had been included, the results might have been different. Another point to consider is that the tears might not show the condition of the ocular surface. The impression cytology of the patients with DED not only showed inflammatory cell infiltration but also showed changes in surface tissue, such as corneal keratinization. The small amount of tears as well as the pathologic changes of the ocular surface could influence IL-17A and IFN-γ levels. Further studies involving larger numbers of tissue analyses via impression cytology or conjunctival biopsy are required. Although we analyzed the individual verification by qPCR, our sample numbers were too small to confirm whether the levels of IL-17A and IFN-γ increased or decreased in patients with DED. We conclusively showed that these cytokines were elevated in Sjögren's DED, whereas non-Sjögren's DED might have a different pathophysiology, at least in the mild and early stages of DED. Finally, the Luminex technology has the potential weakness of protein aggregation through

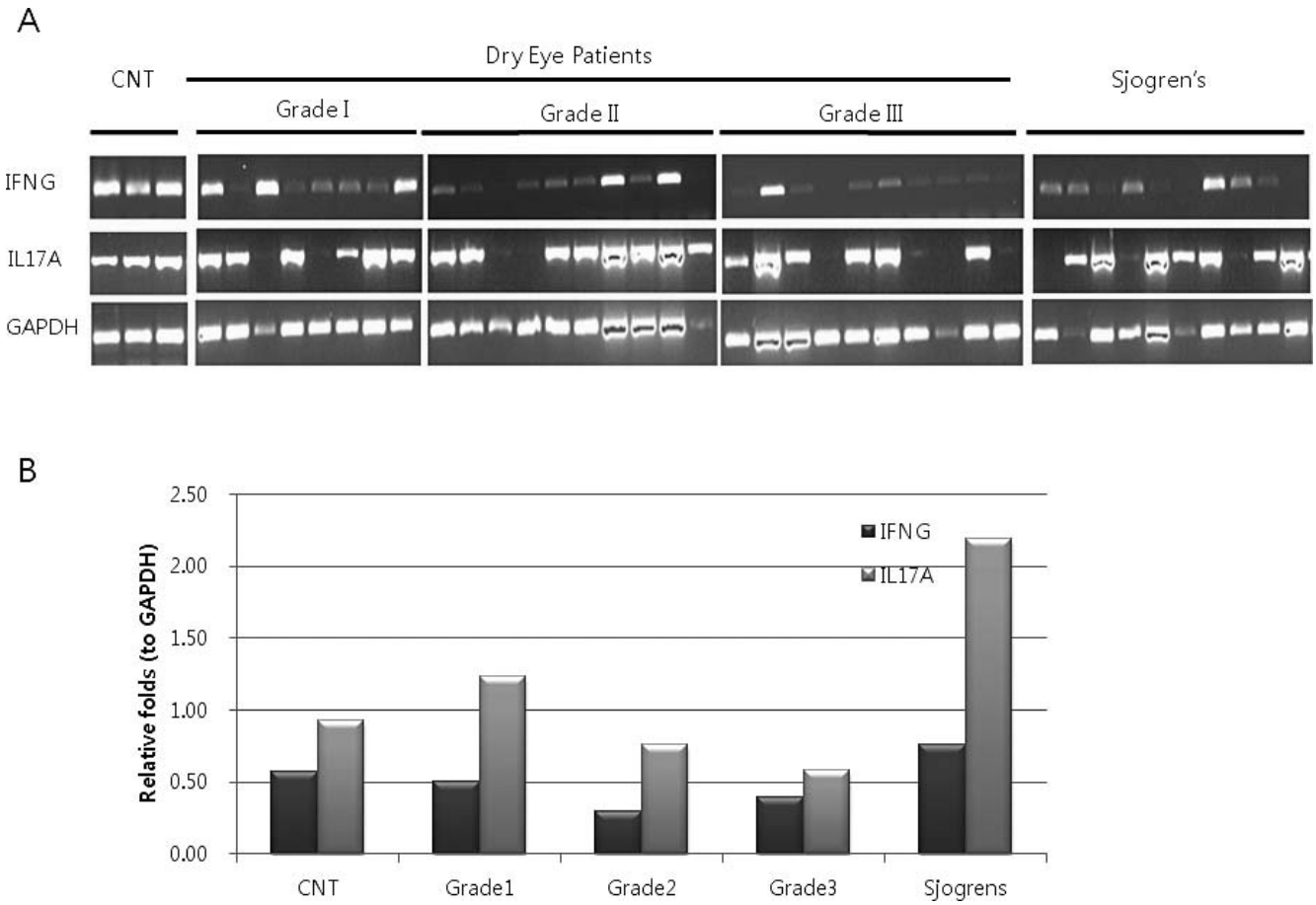

Figure 5. (**A**) Photograph of qPCR result of IFN-γ and IL-17A expression. (**B**) Expression of IFN-γ and IL-17A detected by qPCR. The horizontal axis shows the healthy controls ($n = 3$), dry eye severity 1 ($n = 10$), 2 ($n = 10$), 3 ($n = 10$), and Sjögren's patients with dry eye disease ($n = 10$). Related folds to GAPDH are expressed as the median. Note that the levels of IFN-γ and IL-17A are elevated in Sjögren's patients with DED, but no differences in patients with DED compared with the controls.

protein interactions, which may affect the Il-17A and IFN-γ levels in the tears of patients with DED.

EGF produced by human lacrimal glands has presumed biologic activity on the ocular surface. Decreased EGF is also noted in patients with DED compared with normal controls.[13] EGFR belongs to the receptor tyrosine kinase family and is widely expressed in the ocular surface. The activation of EGFR is involved in the physiologic procedures of cell proliferation, migration, differentiation, and apoptosis via multiple signal transduction pathways, particularly mitogen-activated protein kinases (MAPKs).[41,42] MAPKs are known to stimulate the production of inflammatory cytokines and matrix metalloproteinases (MMPs); they could play important roles in the induction of these factors, which are implicated in the pathogenesis of DED.[43] Increased tear osmolarity causes a signaling cascade that involves phosphorylation of the stress-activated MAPKs, p38, and c-Jun N-terminal kinase (JNK),[44] followed by the activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB); this results in increased levels of proinflammatory cytokines such as IL-1β, TNF-α, IL-8, and IL-6.[12,42] The distinct elevation of proinflammatory cytokines such as IL-6, G-CSF, sEGFR and chemokines, MIP-1δ, in our study suggest that the MAPK pathway is involved in the chronic inflammation of DED pathogenesis.

In the current study, we found increased inflammatory cytokines and chemokine such as CXC3LC1, which is stimulated by proinflammatory cytokines, and decreased anti-inflammatory cytokines in the tears of patients with DED. In conclusion, we found that the levels of some cytokines, chemokines, and soluble receptors are altered in the tears of patients with DED. The concentrations of these molecules are correlated with clinical severity. Many of the molecules increased gradually and significantly according to clinical severity, which was confirmed with classical diagnostic tools. The concentrations of the IL-1/sIL-R1 and IL-6/sIL-6R complexes and their natural inhibitors suggest that these molecules are not merely a consequence of events in the corneal epithelium, but are direct contributors in the development of DED. Compared with previously published studies on inflammatory cytokines in tears of patients with DED, this study differs in that it addresses tear cytokines that appear before the severe stage of DED. Overall, these findings are also beneficial to the development of therapeutic treatment targets using immune-suppressing agents blocking inflammatory pathways, expected for further evaluation.

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