Evaluations of Tear Protein Markers in Dry Eye Disease: Repeatability of Measurement and Correlation with Disease

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**Purpose.** We characterized tear protein markers in dry eye disease (DED).

**Methods.** In this prospective study, based on the ocular surface disease index (OSDI) and corneal staining (CS), 95 DED patients (OSDI ≥13) with increasing CS were enrolled into 3 severity groups: DE1 (CS <4), DE2 (CS 4–7), and DE3 (CS >7), while 25 asymptomatic subjects with no CS were enrolled into the control group (OSDI <13 and CS = 0). Tear fluid was collected at day 0 and day 7 visits, and concentrations of 45 protein markers were measured by multiplexed immunoassay.

**Results.** We analyzed 22 control and 80 DED subjects. Among 33 markers detectable, good inter-visit repeatability was observed with 25 markers, with intraclass correlation coefficients (ICC) ranging from 0.85–0.60; ICCs were <0.60 in the other 8. Correlation with clinical measures was found with two markers, with absolute partial correlation coefficients >0.40: Interleukin-1 receptor antagonist (IL-1Ra) and II-8. IL-1Ra and II-8 correlated with conjunctival staining (0.45, P < 0.001 and 0.35, P < 0.01, respectively), and with Schirmer test (−0.58 and −0.42, P < 0.001). IL-1Ra and II-8 in DE3 were 4.4- and 2.1-fold higher than in DE1 (P = 0.0001 and 0.0007), and 1.9- and 1.6-fold higher than in DE2 (P = 0.022 and 0.017). IL-1Ra in DE2 was 2.5-fold higher than in DE1 (P = 0.038).

**Conclusions.** Tear levels of many immune mediators were highly repeatable between visits in DED. Among them, IL-1Ra and II-8 were associated with clinical signs and disease severity defined by corneal staining. (Invest Ophthalmol Vis Sci. 2012; 53:4556–4564) DOI:10.1167/iovs.11-9054

Dry eye disease (DED) is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface. DED is a highly prevalent condition that affects 5% or more people of various ages in the United States, and the ocular discomfort, irritation, and blurred vision often experienced by dry eye patients can affect daily activity and work-related tasks, thus leading to reduced quality of life.

Symptoms of ocular discomfort or irritation felt by patients generally is the first step of diagnosis of DED and, currently, disease severity is classified by certain clinical objective measurements, such as corneal staining (CS) or tear break up time (TBUT).

Significant progress has been made towards identifying and characterizing the underlying inflammation on the ocular surface in DED through clinical and preclinical research, which has led to better understanding of the key role of inflammation in DED pathogenesis. Different methodologies have been used to collect ocular specimens from patients, and to quantify the molecular and cellular components involved in ocular surface inflammation and in other biologic or pathologic aspects of DED. Up-regulated cell surface expression of human leukocyte antigen (HLA)-DR, intercellular adhesion molecule-1 (ICAM-1) and chemokine receptor type 5 (CCR5) in conjunctival epithelial cells extracted from conjunctival impression cytology specimens obtained from patients with DED were detected using flow cytometry. Higher concentrations of cytokines and chemokines, such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, and matrix metalloprotein-9 (MMP-9), were detected in tear fluids collected from patients with DED, using immunoassays. Increased RNA transcripts of cytokine genes, such as IL-1β, TNF-α, IL-6, MMP-9, and CR5, were detected by real time PCR in conjunctival epithelial cells collected by conjunctival impression cytology from patients with Sjögren syndrome or DED.

Efforts at delineating underlying inflammatory mechanism in DED have led to discovery of new targets and pathways for potential therapeutic intervention for the treatment of DED. However, the lack of precision in standard clinical objective measures, the lack of a "gold standard" for defining disease severity, poor correlation between signs and symptoms, and the significant heterogeneity of patient population in DED remain major challenges in DED clinical research and drug development.

Biomarkers are becoming increasingly important in disease diagnosis and drug development because they potentially allow a more standardized, objective, and precise measurement of a disease and/or treatment response to therapy. Biomarker research in ocular diseases, involving characterization of molecular and cellular components, is important in advancing our understanding of disease and developing therapeutics. However, it also is challenging because of limited access to patient ocular specimens. Cell surface expression of HLA-DR, as a biomarker for ocular surface inflammation, was incorporated into a phase 3 clinical trial with Restasis in DED, and was shown to decrease significantly after 3 and 6 months of treatment with Restasis. More recently, HLA-DR was shown to decrease in two groups treated with tofacitinib (a JAK inhibitor) in a phase 2 trial in DED. Given the known challenges in DED diagnosis, treatment, and clinical drug development, there is significant interest across the dry eye
field in identifying biomarkers that are reliable, robust, and sensitive, and that can be used for DED diagnosis, monitoring drug treatment effect, or stratifying patient populations.

Identifying protein biomarkers in tear fluid is desirable because of easy access to tear fluid. A number of protein markers in tear fluid have shown promise as potential biomarkers for DED. However, it is important to identify and qualify biomarkers analytically and clinically. Different types of biomarkers may have their own qualifications and analytical challenges, depending on their applications (e.g., disease severity diagnoses, mechanistics, for monitoring treatment response, or for patient stratification in a clinical trial).

The objectives of our prospective methodology study were to evaluate the analytic reliability of a large number of protein markers in tears, and to assess whether and which of these markers correlate with clinical measurements and disease severity for DED. Using microcapillary tubes for tear fluid collection and microsphere-based multiplexed immunosassays for measuring concentrations of protein markers in tears, we assessed repeatability of measurements between two different visits, the consistency of protein marker concentrations between fellow eyes, and the correlation of tear protein markers with DED clinical measures and disease severity in groups of control subjects and DED patients with different disease severity.

**MATERIALS AND METHODS**

**Study Design and Evaluations**

The study was conducted in two clinical centers in the United States (Sall Medical Research Center, Inc., Artesia, CA and Total Eye Care, PA., Memphis, TN) as a prospective, non-interventional study with a total enrollment of approximately 120 subjects. The study was conducted in compliance with the ethical principles originating in or derived from the Declaration of Helsinki, and the study protocol was approved by the Institutional Review Boards and/or Independent Ethics Committee of each study center. All subjects provided written informed consent. At screening visit, the subjects were stratified into 4 groups (approximately 30 subjects per group): asymptomatic control subjects, and subjects with mild, moderate, or severe dry eye disease, based on ocular surface disease index (OSDI) and the sum of CS scores (National Eye Institute [NEI] Scale). The control subjects selected were those with an OSDI score <13 and no corneal staining; the DED subjects were those with OSDI scores no less than 13 and were stratified further into a mild dry eye (DE1) group with a CS score <4, a moderate (DE2) group with a CS score of 4–7; and a severe (DE3) group with a CS score greater than >7. For each subject, the eye with the worse CS score was chosen as the study eye and was used in grouping. Each clinical center enrolled approximately 15 subjects of each group.

The major inclusion/exclusion criteria were as follows: subjects enrolled in the control group could not have a history of regular artificial tears usage or self-reported dry eye symptoms over the past 6 months. Subjects were excluded from the study if they had active ocular infection or active allergic conjunctivitis, treatment with an investigational drug or device within 30 days preceding screening visit study procedures and during the study, or use of any medication indicated to increase tear production in subjects whose tear production is presumed to be suppressed due to ocular inflammation, including topical ophthalmic cyclosporine (i.e., Restasis), topical ophthalmic steroids or topical NSAIDS within 30 days preceding the screening visit and during the study. The following also was listed in the exclusion criteria: sporadic use of oral medications designed to treat blepharitis, including doxycycline, tetracycline, oral fish oil, and oral flax seed oil within 1 month of enrollment and during the study (stable use of oral medications for at least one month before study start was permitted); or sporadic use of any medications that could contribute to risk of dry eye, such as beta-blockers, diuretics, or systemic anticholinergic medications (i.e., antihistamines and antidepressants, stable use for at least one month before study start was permitted).

At day 0 visit, which was scheduled 1–3 days post screening visit, eligible subjects were evaluated with patient reported dry eye symptom questionnaires and clinical measurements in the order listed here: OSDI, ocular comfort index (OCI), NEI visual functioning questionnaires (NEI-VF-Q25), tear osmolarity (TearLab, San Diego, CA; average of 3 repeats), Schirmer without anesthesia, TBUT (average of 3 repeats), CS, conjunctival lissamine staining (Oxford scale), and Schirmer test with anesthesia. Capillary tube tear collection was conducted before TBUT and after Schirmer test without anesthesia. At day 7 visit, 6–8 days post day 0 visit, the same assessments were conducted, and tears were collected in the same order and manner as in day 0 visit.

**Biomarker Assessments and Experimental Procedures**

Non-stimulated tear fluids were collected from the tear lake inside the lateral conjunctival sac of the inferior fornix with microcapillary tubes (5 μL, Drummond Scientific, Broomall, PA). dispensed into an Eppendorf microtube (Protein LoBind, VWR, Batavia, IL), and frozen immediately at −70°C. At the end of the study, all tear samples were analyzed for concentrations of 43 protein analytes using microsphere-based immunoassay (Luminex, Austin, TX) in 3 multiplex panels and lysozyme assay. Briefly, microtubes first were centrifuged and tear fluid of precise volume from each sample was transferred to a new tube containing PBS sample buffer (with 4% BSA, 20% fetal calf serum [FCS], and 0.01% SDS) and undergoing analysis using standardized assay procedures. Final dilution factor was 1:12 for 2 multiplexes of cytokines, 1:25,000 for Ig panel and 1:1,000,000 for lysozyme simplex. For tear samples with <3 μL but at least 1 μL available, higher dilution (up to 36-fold for cytokine panels) was used. Tear samples with <1 μL tear fluid available were excluded from analysis. The concentration of each protein analyte was determined from standard curves.

**Statistical Analysis**

Tear protein markers with undetectable levels (below the least detectable dose [LDD] as set by the standard) or missing values in >25% samples for an analyte were excluded from further analysis. The missing value for any remaining protein marker was imputed as either the minimum of the non-missing values for that marker or half of the LDD, whichever was smaller. Protein marker concentration data were log-transformed (log 10) before conducting correlation analyses and statistical comparisons.

For each protein marker measurement, repeatability between visits and consistency between fellow eyes was evaluated using the intraclass correlation coefficient (ICC). ICC was calculated as the ratio of between-subject variance over the sum of between-subject and within-subject variances that were estimated via a linear mixed model with subject as random factor. For each protein marker at day 0 visit, analysis of covariance (ANCOVA) was conducted to assess the effect of different factors, and the model contained terms for sex, age, and study site, along with subject group, and the possible interaction between subject group and study site. The correlation between protein marker level and clinical measures was evaluated using partial correlation coefficient calculated from linear regression analysis controlling potential confounding effect of age, sex, and study site in the study. ICC and regression analyses were conducted first using data from all subjects, then from dry eye subjects.

Linear mixed model of variance analysis was used to determine if levels of each protein marker differed between subject groups. The model included age, sex, study sites, and disease groups as fixed independent variables, and visits nested within subject as random effects. Post-hoc comparisons between subject groups were performed by general linear hypothesis and P values were estimated without...
multiplicity adjustment due to the exploratory nature of the study. Statistical significance was set at level of 0.05, all P values are 2-sided.

RESULTS

Patient Demographics and Clinical Assessments

A total of 95 DED patients and 25 asymptomatic control subjects was enrolled in the study. Tear protein marker results described are from tear samples obtained from 80 DED patients and 22 asymptomatic control subjects. Demographic and clinical features of each DED group and control group at screening visit are summarized in Table 1. We excluded tear samples with volume <1 μL (27 of 446 total samples, ~6%), specifically, 2 subjects from the DE1 group, 5 from DE2, 8 from DE3, and 3 from the control group.

DED groups had a higher OSDI total score and corneal staining than the control group as expected, since they were used to stratify between asymptomatic controls with no conjunctival lissamine green staining and Schirmer test with anesthesia were present, as expected, since they were used to stratify between DED groups (Figs. 1C, 1F, 1I, 1L). The half-length of the 95% limits of agreement in Bland-Altman plot is the repeatability coefficient (RC) for visit-to-visit, and it is a precision measure for a given test method. RC around 3.8 was observed for IL-15 and IL-8, and around 5 for IL-1Ra, compared to an RC >20 for AAT, which showed a lower ICC in visit-to-visit comparisons (Table 2).

Taken together, our data showed that, of the many immune mediators in tear film, consistent levels were detected in fellow eyes and over consecutive visits seven days apart. These results confirm the technical reliability of the methodologies and assay platforms used to quantify these markers in tears.

Measuring Protein Markers Concentration in Tears: Repeatability between Visits and Correlation between Fellow Eyes

Among the 43 tear protein analytes assayed, 31 had detectable levels in more than 75% of samples and, thus, were included in subsequent statistical analysis. ICCs of 31 protein markers between visits (days 0 and 7), and between study eye and non-study eyes of subjects are listed in Table 2.

ICCs >0.60 were found in 25 markers in study eyes between visits, 0.30–0.60 in 7 markers, and <0.30 in 1 marker; very similar ICCs were found in non-study eyes (Table 2). Similar ICCs between study and non-study eyes also were observed for most markers in tears. IL-6 and IL-18, together with IgA, IgM, apolipoproteins (A1, H, and CIII), and alpha-1-antitrypsin (AAT) showed lower ICCs than most other immune mediators and protein markers in the study. IgA, IgM, different apolipoproteins and AAT; known major protein components of plasma, were in greater concentration (mg/mL) in tears than most of the cytokines and chemokines in tears (ng/mL or pg/mL, data not shown).

Scatter plots of eye-to-eye and visit-to-visit of four representative protein markers are shown in Figure 1, with two ICCs listed in each graph: one based on all study subjects and one based on DED patients only are shown. ICCs based on DED patients generally were similar to ICCs based on all subjects. Visit-to-visit repeatability of these four markers was illustrated further with Bland-Altman plots (Figs. 1C, 1F, 1I, 1L). The half-length of the 95% limits of agreement in Bland-Altman plot is the repeatability coefficient (RC) for visit-to-visit, and it is a precision measure for a given test method. RC around 3.8 was observed for IL-15 and IL-8, and around 5 for IL-1Ra, compared to an RC >20 for AAT, which showed a lower ICC in visit-to-visit comparisons (Table 2).

Taken together, our data showed that, of the many immune mediators in tear film, consistent levels were detected in fellow eyes and over consecutive visits seven days apart. These results confirm the technical reliability of the methodologies and assay platforms used to quantify these markers in tears.
Figure 1. Eye-to-eye, visit-to-visit scatter plots and Bland-Altman plots of four selected markers in tears. (A–C) Alpha 1 antitrypsin. (D–F) IL-15. (G–I) IL-1Ra. (J–L) IL-8. (A, D, G, J) Eye-to-eye scatter plots. (B, E, H, K) Visit-to-visit scatter plots. The 2 ICCs within parenthesis are based on all subjects and dry eye patients only, respectively. (C, F, I, L) Visit to visit Bland-Altman plots. With tear protein data Log transformed before analysis, geometric mean and ratio are shown in x and y axes, respectively. Dashed lines: mean of the differences and 95% limits of agreement. Black: control. Green: DE1. Blue: DE2. Red: DE3.
Correlations of Tear Protein Markers with DED Clinical Measurements

We next evaluated correlations between each marker and dry eye symptoms, and clinical objective measurements using partial correlation analysis, controlling for age, based on all four groups of subjects in the study, as well as based on only DED patients, respectively. All absolute partial correlation coefficients were <0.70 between tear protein markers and DED symptoms measured by OSDI total score, OCl score or NEI-VFQ-25 composite score, and clinical objective measures, with the best and most consistent correlations found between clinical objective measures and IL-1Ra, followed by IL-8 (partial correlation coefficients >0.40, \( P < 0.01 \)). The partial correlation coefficients of IL-1Ra and IL-8 with clinical measures at day 0 are listed in Table 3. Based on all study subjects, partial correlations between IL-1Ra and Schirmer test (with and without anesthesia) were −0.619 and −0.496, respectively, in study eyes at day 0, indicating inverse correlation. They were −0.448 and −0.585, respectively, in study eyes on day 7, and −0.505 and −0.496, respectively, in non-study eyes on day 0 (data not shown, \( P < 0.001 \)). IL-1Ra in tear also was found correlated with corneal staining in DED patients (0.414, \( P < 0.001 \), Table 3). Similar levels of correlations with corneal staining also were observed on day 7 (data not shown). As shown in Table 3, IL-8 had similar correlations with clinical measures as IL-1Ra, but slightly weaker. No other tear protein markers showed comparable correlation with DED clinical assessments (absolute partial correlation coefficient >0.40) in this study. Our observation that IL-1Ra and IL-8 associate with DED clinical objective measures led us to examine whether they associate significantly with DED severity.

Differential Expression of Tear Markers in Relation to Dry Eye Disease Severity

Group (geometric) means, 95% confidence intervals (CI), and range of IL-1Ra and IL-8 at each visit are listed in Table 4. Group difference was assessed with a linear mixed model using data from both visits, with subject group, age, sex, and study site included as fixed factors. \( P \) values from post hoc pair-wise comparisons between groups from the linear model are shown in Figure 2, under each bar graph showing the group (geometric) means and 95% CIs of IL-1Ra and IL-8, together with corneal staining and conjunctival staining scores. As expected, each group was significantly different in corneal staining, with higher staining score in more severe group. A similar pattern also was seen in conjunctival staining, except that it was not significantly different between the normal and DE1 groups (\( P = 0.272 \)). Tear levels of IL-1Ra and IL-8 showed significant differences among DED patient groups, with highest levels found in DE3 and lowest in DE1. IL-1Ra in the DE3 group was 4.4-fold (\( P = 0.0001 \)) and 1.9-fold (\( P = 0.022 \)) higher than in DE1 and DE2, respectively. IL-1Ra in the DE2 group was 2.3-fold higher than in DE1 (\( P = 0.038 \)). IL-8 in DE3 was 2.1-fold (\( P = 0.0007 \)) and 1.6-fold (\( P = 0.017 \)) higher than in DE1 and DE2.

To explore and compare criteria for severity stratification used in other dry eye studies, we conducted post hoc analysis of subject groups by adding the following criteria for subject group classification: OSDI <13, CS = 0 and TBUT >7 for control; OSDI \( \geq \)20 and TBUT \( \leq \)7 for all dry eye patients, and removing those not satisfying these criteria from the post hoc analysis (removing 14 from control; and 8, 3, and 1 from DE1, DE2, and DE3, respectively). It should be noted that the 14 subjects who were excluded from the control group post hoc

### Table 3. Partial Correlation Coefficients of IL-1Ra and IL-8 with Dry Eye Clinical Signs in Study Eye and Symptom Measured by Ocular Surface Disease Index (OSDI) at Day 0 Visit among All Subjects and Dry Eye Patients Only, Respectively

<table>
<thead>
<tr>
<th>Test</th>
<th>All Subjects (( N = 102 ))</th>
<th>Dry Eye Subjects (( N = 80 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1Ra</td>
<td>IL-8</td>
</tr>
<tr>
<td></td>
<td>Geometric Mean (95% CI)</td>
<td>Range</td>
</tr>
<tr>
<td>Corneal staining</td>
<td>0.224 (0.159)</td>
<td>0.414* (0.279)</td>
</tr>
<tr>
<td>Conjunctival staining</td>
<td>0.370† (0.315†)</td>
<td>0.425* (0.354†)</td>
</tr>
<tr>
<td>Schirmer w/o anesthesia</td>
<td>−0.496* (−0.368*)</td>
<td>−0.479* (−0.377*)</td>
</tr>
<tr>
<td>Schirmer w/ anesthesia</td>
<td>−0.619* (−0.425*)</td>
<td>−0.583* (−0.418*)</td>
</tr>
<tr>
<td>TBUT</td>
<td>−0.076 (−0.103)</td>
<td>−0.066 (−0.116)</td>
</tr>
<tr>
<td>Tear osmolarity</td>
<td>0.117 (0.058)</td>
<td>0.269 (0.110)</td>
</tr>
<tr>
<td>OSDI total score</td>
<td>0.014 (−0.037)</td>
<td>0.117 (0.025†)</td>
</tr>
</tbody>
</table>

w/o, without; w/, with.

* \( P < 0.001 \).

† \( P < 0.01 \).

### Table 4. Group Geometric Mean, 95% CI and Range of IL-1Ra and IL-8 at Day 0 and Day 7 Visits, Respectively

<table>
<thead>
<tr>
<th>Group (IL-1Ra ng/mL)</th>
<th>Geometric Mean</th>
<th>95% CI</th>
<th>Range</th>
<th>Geometric Mean</th>
<th>95% CI</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>325.2</td>
<td>196.4, 538.6</td>
<td>58.6–2306.9</td>
<td>288.1</td>
<td>173.2, 479.1</td>
<td>37.3–3565.0</td>
</tr>
<tr>
<td>DE1</td>
<td>205.1</td>
<td>138.2, 304.3</td>
<td>28.7–2135.0</td>
<td>259.0</td>
<td>175.3, 382.9</td>
<td>48.6–2030.0</td>
</tr>
<tr>
<td>DE2</td>
<td>434.5</td>
<td>287.1, 657.6</td>
<td>8.1–2265.7</td>
<td>616.1</td>
<td>416.0, 910.7</td>
<td>31.4–2599.4</td>
</tr>
<tr>
<td>DE3</td>
<td>930.73</td>
<td>569.5, 1521.2</td>
<td>111.1–4286.4</td>
<td>1096.7</td>
<td>666.0, 1806.0</td>
<td>84.0–5525.6</td>
</tr>
</tbody>
</table>

IL-8 (ng/mL)

| Control              | 4.60          | 3.4, 6.22 | 1.4–20.9 | 3.77          | 2.82, 5.04 | 1.5–19.4 |
| DE1                  | 3.31          | 2.67, 4.09 | 1.3–14.8 | 3.89          | 2.96, 5.12 | 1.4–38.5 |
| DE2                  | 5.38          | 3.97, 7.31 | 0.9–28.2 | 6.07          | 4.17, 8.84 | 0.7–106.4 |
| DE3                  | 9.73          | 5.7, 16.6 | 1.5–203.9 | 8.19          | 4.57, 14.69 | 1.3–171.4 |

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933262/ on 10/17/2017
had OSDI scores <13 and no corneal staining. As shown in Figure 3, compared to prospectively defined groups, very similar patterns and significant differences were found among the groups in corneal staining, conjunctival staining, IL-1Ra, and IL-8, except that the control group had fewer subjects, and lower levels of IL-1Ra and IL-8 (N = 8, 160, and 3.1 ng/mL, respectively). In our analysis, IL-1Ra in the post hoc control group now is significantly lower than in DE3 group (P = 0.029, Fig. 3C).

**DISCUSSION**

In our large multicenter methodology study with controls and DED subjects, we characterized comprehensively a large
number of protein markers, mostly immune mediators, in tears and found that concentrations of many of them were highly repeatable between 2 visits 7 days apart, and were highly consistent between the fellow eyes. Among 31 markers detectable in our study, we found that IL-1Ra and IL-8 correlated best with DED clinical signs and disease severity.

To our knowledge, this study is the first examining the repeatability of measuring different protein concentrations in tears from the same eyes prospectively in DED patients and control subjects.15–19,24 There were many known challenges involving ocular specimen collection/handling at different clinical sites, overcoming the limits of tear volume from moderate to severe aqueous deficient DED patients, and obtaining reliable analytical data from limited quantity of tear fluid. Our findings of good repeatability and consistency in tear levels of many immune mediators indicated that the methodologies offer technical reliability and robustness for potential clinical applications. Perhaps more importantly, our findings

![Figure 3](image_url)
Evaluation of Tear Protein Markers in Dry Eye

indicated that inflammation in DED occurs bilaterally, and that the profile of immune mediators in tear film generally is stable.

The tear IL-1Ra concentrations we detected (around 220–300 ng/mL in the control and mild DED groups, and 500 and 1000 in the moderate and severe DED groups, respectively) are highly comparable to the tear IL-1Ra concentrations measured by ELISA reported previously (295 ng/mL in tears from normal eyes, 940 in eyes with meibomian gland disease and 2400 ng/mL in eyes of Sjögren syndrome patients).15 and are consistent with a previous report of higher level of IL-1Ra in evaporative DED than in normal eyes. Here, we further demonstrated association between IL-1Ra level and DED disease severity. Taken together, it appears that the concentration of IL-1Ra in tears has high abundance (high ng/mL); good reliability analytically; good reproducibility between different studies in DED patients of different etiology; a wide dynamic range from mild-to-moderate, moderate-to-severe DED groups (2- to 4-fold); and association with DED disease severity defined by ocular surface damages.

IL-1Ra is a naturally occurring and specific inhibitor of IL-1 activity. It can bind to IL-1 receptor 1 tightly without triggering inflammatory signaling and, thus, antagonize inflammatory effects induced by IL-1β and IL-1α. IL-1Ra often is produced abundantly in inflamed tissues, and thought to be an endogenous regulatory mechanism against IL-1 mediated inflammation and tissue damage. Human intestinal epithelial cells are the major source of IL-1Ra in the mucosa and upon inflammation they respond with an up-regulation of IL-1Ra production. In ocular tissues, IL-1Ra is expressed abundantly in normal corneal and conjunctival epithelium, and the strongest expression is in the superficial apical layer of corneal epithelium. Numerous studies have shown anti-inflammatory and tissue protective benefits by IL-8a in different diseases or inflammatory conditions. It is very likely that the higher level of IL-1Ra in DED patients with more severe ocular surface damage is secondary to ocular surface inflammation and the resultant release of inflammatory cytokines, which in turn may induce higher expression of IL-1Ra in the ocular epithelial cells.

Our findings of a significantly higher level of IL-8 in tears of severe DED group as defined by corneal staining are consistent with previous reports of tear IL-8 correlating with corneal and conjunctival stainings. IL-8 is one of the major mediators of the inflammatory response and is released from several cell types, including macrophages and epithelial cells, in response to an inflammatory stimulus. It was reported that over-expression of IL-8 in the cornea induces ulcer formation in the severe combined immunodeficiency (SCID) mouse. Unlike anti-inflammatory IL-1Ra, IL-8 is proinflammatory and a chemotactic factor for neutrophils. Thus, increased tear level of IL-8 could contribute further to ocular surface inflammation and, subsequently, more epithelial damage in DED.

Despite a large number and a wide range of DED patients in our study and the evaluation of a large number of immune mediators in the analysis, we found only weak-to-moderate correlations between dry eye clinical signs and tear markers, based on the partial correlation coefficient (<0.70). Others have reported previously correlation of protein markers, such as MMP-9 and IL-6, with DED clinical measures based on Spearman rank correlation. We did not find other markers here with an absolute partial correlation coefficient >0.40 with DED measures, beside IL-1Ra and IL-8. Given the well-known discordance between different dry eye clinical signs, and between signs and symptoms, this lack of strong correlations between tear markers and clinical assessments is not surprising. This could be due to a number of factors, including different DED patient populations in different studies, methodology of sample collection and analysis, as well as different method of data analysis. In our study, the patient selection criteria were based on symptom and corneal staining scores, straightforward yet covered a continual spectrum of patients with and without corneal epithelial abnormality. Other studies used combinations of 3–5 criteria, which tend to select discrete populations and exclude many patients. One cannot exclude the possibility of selection bias in our study or in other studies, which in turn could lead to different levels of correlations between parameters measured in the studies. Method and technique for tear collection and cytokine analysis also are varied among studies, for example, different multiplex kits and sample dilution buffer (matrix effect) could result in different tear cytokine concentrations. Partial correlation coefficient, calculated from linear regression analysis, and controlling the potential confounding effect of age, sex, and other factors, was used in our study to evaluate the strength of linear relationship between tear markers and clinical parameters, while others have used Spearman’s rank correlation. In general, it is thought that partial correlation measures more accurately the association between 2 variables than Spearman rank correlation, because it adjusts for other potential confounding variables (e.g., age factor in our study).

We hypothesized that the discordance in dry eye between symptoms and clinical signs, and between biomarker and clinical parameters is due likely to the significant heterogeneity of DED patient populations in which multiple subtypes may have different underlying pathophysiologic mechanisms or genetic backgrounds that lead to different clinical manifestations. Thus, biomarker research in DED directed towards characterizing different patient populations may provide insight into the mechanisms of pathogenesis or pathophysiologic of the disease, and identify tools for addressing challenges of DED discordance in clinical signs and symptoms.

There are a number of limitations in our study. Obtaining tear fluid from the severe aqueous-deficient dry eye patients who have very little tear is a challenge. Thus, results from tear marker analysis in dry eye are likely to be biased towards a non-severe aqueous-deficient population. The stratification in our study did not take into consideration the different etiologies and subtypes. Age was imbalanced between control and dry eye subjects in our study; however, age factor was adjusted in the statistical analysis when calculating partial correlation coefficient from linear regression and determining group difference in tear markers using a linear mixed model. Furthermore, we calculated and compared ICCs and correlation coefficients based on all subjects as well as DED patients only, and results were similar (Table 3 and Fig. 1). There also were more female subjects in the study, similar to the demographics of dry eye disease populations. However, in our analysis of covariance examining age, sex, study site, and group effect, we found sex had no significant effect on tear level of IL-1Ra (P = 0.810) or IL-8 (P = 0.252).

In summary, our study shows good repeatability and reliability of measuring many immune mediators in the tears of DED patients. Among them, IL-1Ra, and to a lesser extent IL-8, correlate with dry eye clinical signs and disease severity defined by corneal staining. Thus, our study opens venues to further investigations regarding other markers in tears in DED or in other ocular surface diseases, and the potential use of tear IL-1Ra and IL-8 as biomarkers for DED.

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References


