Tear Cytokine Profile as a Noninvasive Biomarker of Inflammation for Ocular Surface Diseases: Standard Operating Procedures

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PURPOSE. To provide standard operating procedures (SOPs) for measuring tear inflammatory cytokine concentrations and to validate the resulting profile as a minimally invasive objective metric and biomarker of ocular surface inflammation for use in multicenter clinical trials on dry eye disease (DED).

METHODS. Standard operating procedures were established and then validated with cytokine standards, quality controls, and masked tear samples collected from local and distant clinical sites. The concentrations of the inflammatory cytokines in tears were quantified using a high-sensitivity human cytokine multiplex kit.

RESULTS. A panel of inflammatory cytokines was initially investigated, from which four key inflammatory cytokines (IL-1 β , IL-6, INF- γ , and TNF- α) were chosen. Results with cytokine standards statistically satisfied the manufacturer's quality control criteria. Results with pooled tear samples were highly reproducible and reliable with tear volumes ranging from 4 to 10 μ L. Incorporation of the SOPs into clinical trials was subsequently validated. Tear samples were collected at a distant clinical site, stored, and shipped to our Biomarker Laboratory, where a masked analysis of the four tear cytokines was successfully performed. Tear samples were also collected from a feasibility study on DED. Inflammatory cytokine concentrations were decreased in tears of subjects who received anti-inflammatory treatment.

CONCLUSIONS. Standard operating procedures for human tear cytokine assessment suitable for multicenter clinical trials were established. Tear cytokine profiling using these SOPs may provide objective metrics useful for diagnosing, classifying, and analyzing treatment efficacy in inflammatory conditions of the ocular surface, which may further elucidate the mechanisms involved in the pathogenesis of ocular surface disease.

Keywords: dry eye disease, tear cytokine profile, milliplex assay, standard operating procedures

Cytokine profiling of body fluids has become key in the diagnosis, classification, and evaluation of inflammatory diseases.^{1–3} Monitoring cytokine changes and correlating them with signs and symptoms will improve our understanding of the immune mechanisms. Ocular surface diseases are often associated with inflammation.^{4–6} However, with many ocular diseases, such as dry eye disease (DED), only small volumes of nonstimulated tear samples are obtainable. To reliably quantify tear cytokines, standard operating procedures (SOPs) must be established and validated. Only then can tear cytokine profiling be incorporated into multicenter clinical trials to evaluate changes over time and across trials. Such metrics may also provide information on disease mechanisms and new approaches to treatment.

Dry eye disease is a multifactorial ocular surface disease often associated with inflammation.^{7–9} The inflammatory mediators associated with DED pathogenesis can be categorized as ubiquitous inflammatory cytokines,^{10,11} Th1-related cytokines,^{12–14} Th17-related cytokines,^{14–17} chemokines and their receptors,^{14,18–22} metalloproteinase,^{21,23} and secretory phospholipases.^{24–26} Previous studies on DED demonstrated changes in tear cytokine expression in both humans^{10,13,15,16,24}

and animals,^{27–31} allowing their use as a minimally invasive objective biomarker to classify severity, assess treatments, and elucidate mechanism of the disease. However, published data display significant variability (see Supplementary Table S1).^{14–16,22,32–34} Such variability could reflect biological differences among subjects as well as differences in methodology. Since variability distorts the interpretation and comparison of clinical trial outcomes, establishing SOPs will provide reliable information on tear cytokine changes.

Luminex technology (Luminex Corporation, Austin, TX) is currently one of the most frequently used cytometric bead-based methodologies for biological applications,^{11,12,14,16,17,32,33,35–57} capable of simultaneously quantifying multiple targets in a single 50- μ L sample with high sensitivity. To integrate tear cytokine profiles as a noninvasive objective biomarker of DED, one of the major concerns is the smaller sample volumes and potentially lower concentrations available as compared to other biological fluids, making repeat measurements unlikely, and interference between sample matrix and assay beads are common.^{35,40,58} It is important to determine whether Luminex/Millipore methodologies can reliably measure tear cytokine concentrations. Also, as standard Milliplex assay protocol requires 50 μ L of sample,

TABLE 1. Summary of Precision and Accuracy Determination With Four Cytokine Standards

	Experiment 1			Experiment 2			Experiment 3			Experiment 4			Interassay, %CV	MinDC, pg/mL
	Detectable Ranges, pg/mL	%CV	R ²	Detectable Ranges, pg/mL	%CV	R ²	Detectable Ranges, pg/mL	%CV	R ²	Detectable Ranges, pg/mL	%CV	R ²		
IL-1 β	0.11–425.52	4.15	0.99	0.06–2136.32	0.74	1.00	0.07–9,575.60	1.48	1.00	0.02–1523.07	0.71	1.00	15.86	0.13
IL-6	0.10–258.28	5.61	0.99	0.11–2302.48	1.17	1.00	0.79–10,967.81	1.45	1.00	0.03–1587.28	1.66	1.00	20.12	1.22
IFN- γ	0.14–1574.98	1.96	0.99	0.07–1991.70	0.95	1.00	0.40–11,101.25	0.95	1.00	0.02–2230.03	0.95	1.00	40.04	1.21
TNF- α	0.02–213.42	0.52	1.00	0.05–3996.88	0.32	1.00	0.08–7,547.66	2.16	0.99	0.04–1681.72	1.03	1.00	39.47	0.10

The detectable ranges were determined by the MILLIPLEX Analyst software. The MinDC was calculated as the mean + 2 SD at input of 0.00 pg/mL of four independent experiments (see Table 2 for details). %CV: intra-assay precision for each of the four experiments. Interassay %CV: interassay precision of the four experiments. R² was determined by the MILLIPLEX Analyst software.

significant tear dilution is inevitable and the proper dilution range must be determined. The purpose of this study was to validate Luminex as a specific potential method for the analysis of tear samples from dry eye subjects.

To choose which cytokines to analyze, a literature review was performed on the roles of cytokines in DED pathogenesis and their availability for Luminex assay analysis. Previous studies indicated that IL-1 β , IL-6, IFN- γ , and TNF- α have frequently displayed changes in tears of DED patients as compared to non-DED patients in same studies or research group in a year (see Supplementary Tables S1, S2).^{14–16,22,32,33,37} Although Luminex assays can be used to quantify hundreds of molecules simultaneously from individual small-volume samples, it is not practical to assay every cytokine in one experiment.

In this study, IL-1 β , IL-6, IFN- γ , and TNF- α were chosen for analysis by Luminex assays for their apparent key roles in the pathophysiology of DED and for their compatibility to fit in a single 50- μ L assay. Because there is no single commercial kit available specifically tailored for tear cytokine analysis, we developed SOPs able to provide a reliable cytokine profile in multicenter randomized clinical trials (RCTs) as a biomarker of inflammation to help better understand the mechanism of the pathogenesis of ocular surface diseases and leading to novel combating strategies.

MATERIALS AND METHODS

Participating Subjects

Institutional Review Board (IRB)-approved informed consents were obtained, and the tenets of the Declaration of Helsinki were followed. Tears were collected from both normal subjects and those considered to have DED per the Definition and

Classification Subcommittee of the International Dry Eye WorkShop.⁹ Signs and symptoms evaluated included the Ocular Surface Disease Index (OSDI) questionnaire, visual acuity assessment, Schirmer tear test, tear breakup time, corneal fluorescein staining, and conjunctival lissamine green staining, as previously described.^{9,32,59}

Tear Sample Collection, Storage, Shipping, and Tracking

Nonstimulated tears were collected from the lower lid margin using Drummond disposable 20- μ L microcapillary tubes (Sigma-Aldrich Co. LLC, St. Louis, MO).^{32,59} Precautions were taken to minimize contact between the capillary tube and the surrounding tissues. Approximately 4 to 5 μ L of tears were collected from each eye in 5 minutes between 11:00 AM and 4:00 PM to minimize diurnal variation. Tears were expelled into precooled, prelabeled, individually packed and sterilized safe-lock Eppendorf tubes, sealed with Parafilm (Fisher Scientific, Inc., Pittsburgh, PA), identified with masked codes utilizing laser-printable Brady labels (Fisher Scientific, Inc.), stored on ice for <30 minutes, and then transferred to –80°C freezers until analysis. Samples collected from a distant clinical site were packed utilizing dry ice and International Air Transport Association-certified Infecon 5000 shipper kits (Fisher Scientific, Inc.), following manufacturer's instructions, and then shipped to the Mount Sinai Biomarker Laboratory on dry ice overnight. Tear samples were transferred to a –80°C freezer without thawing. All coded labels were examined for accuracy, then entered into an encrypted electronic relational database tailored for high throughput. The freezer was connected to an auto-alarm system and an emergency power backup.

TABLE 2. Recovery Assays of Standards

Measured		IL-1 β				IL-6				IFN- γ				TNF- α	
Input	Mean $\pm$ SD, pg/mL	N ^a	% Recovery	Mean $\pm$ SD, pg/mL	N	% Recovery	Mean $\pm$ SD, pg/mL	N	% Recovery	Mean $\pm$ SD, pg/mL	N	% Recovery			
0	0.03 $\pm$ 0.05	8	NA	0.24 $\pm$ 0.44	8	NA	0.25 $\pm$ 0.48	8	NA	0.02 $\pm$ 0.04	8	NA			
0.13	0.08 $\pm$ 0.07	8	62	0.13 $\pm$ 0.17	8	97	0.23 $\pm$ 0.31	8	175	0.09 $\pm$ 0.08	8	72			
0.64	0.66 $\pm$ 0.16	8	103	0.65 $\pm$ 0.15	8	102	0.6 $\pm$ 0.27	8	94	0.74 $\pm$ 0.13	8	115			
3.2	3.39 $\pm$ 0.5	8	106	3.91 $\pm$ 0.52	8	122	3.08 $\pm$ 1.67	8	96	3.46 $\pm$ 0.53	8	108			
16	15.89 $\pm$ 3.67	8	99	14.17 $\pm$ 5.29	8	89	16.09 $\pm$ 5.66	8	101	15.72 $\pm$ 5.72	8	98			
80	72.04 $\pm$ 12.28	8	90	79.16 $\pm$ 21.24	8	99	83.47 $\pm$ 21.52	8	104	97.92 $\pm$ 62.33	8	122			
400	437.23 $\pm$ 66.7	8	109	416.3 $\pm$ 182.4	8	104	401.99 $\pm$ 81.39	8	100	400.49 $\pm$ 164.95	8	100			
2000	2086.21 $\pm$ 674.69	8	104	2149.92 $\pm$ 526.48	8	107	1836.8 $\pm$ 222.8	7*	92	1783.36 $\pm$ 1209.75	8	89			

* Results were calculated from four independent experiments, each with duplicate measures (N = 8). Only one measure of IFN- γ at the 2000 pg/mL point had a value larger than the mean + 2 SD (an outlier), which was excluded from calculation (N = 7).

TABLE 3. A Representative Experiment With Three Quality Controls of Four Cytokines at Known Concentration Ranges

ID	Volume, μ L	IL-1 β			IL-6			INF- γ			TNF- α		
		Expected, pg/mL	Recovery, %	% CV	Expected, pg/mL	Recovery, %	% CV	Expected, pg/mL	Recovery, %	% CV	Expected, pg/mL	Recovery, %	% CV
QC1	50	20–42	100	0.43	22–46	100	7.41	24–50	100	10.82	21–44	100	5.23
QC2	50	154–320	129	0.87	177–367	100	3.06	179–372	100	2.21	168–348	105	3.2
QC3A	50	0.07–0.14	100	19.4	0.42–0.70	103	17.6	3.75–5.23	100	19.12	0.51–0.65	100	17.87
QC3B	50	0.10–0.42	117	21.66	0.60–1.04	107	23.59	4.82–6.96	100	19.92	0.64–0.88	108	17.92

QC1, quality control 1 of standards at lower concentration ranges; QC2, quality control 2 of standards at higher concentration ranges; QC3, aliquot of 10 μ L tears. Measurements that were within the expected ranges were considered as 100% recovery. For the measurements higher than the upper range of the expected, the percent recovery is larger than 100%.

Standard Tear Analysis for Cytokine Determination

Tear samples were thawed on ice and the volumes recorded. To avoid uneven evaporation, samples were kept at -80°C immediately after the volume measurements. Assay buffer was added to each sample up to 50 μ L and loaded to designated wells to initiate the reaction in a 96-well plate of a High Sensitivity Human Cytokine Kit (EMD Millipore Corporation, Billerica, MA) and assayed according to the manufacturer's instructions. Plates were read on MAGPIX System (EMD Millipore Corporation); the median fluorescence intensities (MFI) were recorded, evaluated, and converted into their respective concentrations (pg/mL) using MILLIPLEX Analyst software (EMD Millipore Corporation).

Experimental Protocols

MilliPlex Assay Utilizing Commercial Standards.

Precision, Accuracy, and Sensitivity. Multiple cytokine standard mixtures were serially diluted with assay buffer to yield final concentrations of 2000, 400, 80, 16, 3.2, 0.64, and 0.13 pg/mL, respectively. Diluted standards were assayed in four independent experiments, with duplicate measurements. Coefficient of variation (CV) was utilized to evaluate precision, calculated as standard deviation (SD) divided by the mean, and reported as a percentage (%CV). Intra-assay %CV was generated by MILLIPLEX Analyst software (EMD Millipore Corporation) from reportable results across two different concentrations of one experiment. Interassay %CV was generated from across

two different concentrations (0.64 and 80 pg/mL) of four independent experiments. Accuracy was represented as the mean percentage recovery of three concentration levels of the standards ranging from 16 to 400 pg/mL in assay buffer. Sensitivity was represented as the minimum detectable concentration (MinDC, pg/mL) per manufacturer's manual. MinDC for each experiment was calculated by the MILLIPLEX Analyst software (EMD Millipore Corporation). It measures the true limits of detection by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions. MinDC of four experiments was calculated as mean $\pm$ 2 SD at input of 0.00 pg/mL.

Effect of Dilution of Standards. Variability was represented in the coefficient of multiple correlations (R^2 value). The percentage of recovery at each point was calculated as the ratio (%) of the mean of cytokine concentrations experimentally measured divided by the input.

Batch-to-Batch Repeatability. For clinical trials, the comparability of results from a series of analyses over time must be demonstrated. To achieve this, two quality controls of known concentration ranges, as determined by the manufacturer, were measured in duplicates. QC1 represents a standard of known concentration within the lower concentration range (20–50 pg/mL), while QC2 represents the same within the higher concentration range (100–500 pg/mL). A concentration measured within the expected ranges was considered as 100% recovery.

TABLE 4. Levels of Four Cytokines in Two Pooled Normal Tear Samples

Sample ID	Tear		Number of Repeats	IL-1 β			IL-6			INF- γ			TNF- α		
	Vol., μ L	Dilution Factor		Mean $\pm$ SD	% CV	P Value	Mean $\pm$ SD	% CV	P Value	Mean $\pm$ SD	% CV	P Value	Mean $\pm$ SD	% CV	P Value
A1	2	25	7	$<0.02 \pm 0.01$	18.16	$<0.05^*$	0.36 ± 0.11	17.7	>0.05	3.51 ± 1.34	22.54	>0.05	0.46 ± 0.1	14.75	>0.05
A2	4	12.5	8	0.07 ± 0.08	23.79	>0.05	0.53 ± 0.23	28.18	>0.05	4.8 ± 1.88	27.9	>0.05	0.59 ± 0.19	25.32	>0.05
A3	6	8.3	7	0.1 ± 0.06	19.89	>0.05	0.53 ± 0.11	13.38	>0.05	4.32 ± 1.59	24.9	>0.05	0.59 ± 0.12	15.9	>0.05
A4	8	6.3	7	0.12 ± 0.09	19.06	>0.05	0.68 ± 0.12	12.99	>0.05	5.53 ± 0.71	9.66	>0.05	0.65 ± 0.12	14.5	>0.05
A5	10	5	7	0.14 ± 0.08	16.1	>0.05	0.72 ± 0.16	15.73	>0.05	4.31 ± 0.69	10.61	>0.05	0.63 ± 0.15	18.87	>0.05
SUM			36	0.11 ± 0.03	19.4		0.56 ± 0.14	17.6		4.49 ± 0.74	19.12		0.58 ± 0.07	17.87	
B1	2	25	8	0.03 ± 0.04	16.93	$<0.05^{\dagger}$	0.5 ± 0.16	20.64	$<0.05^{\dagger}$	4.11 ± 1.1	17	$<0.05^*$	0.61 ± 0.15	19.48	>0.05
B2	4	12.5	8	0.25 ± 0.11	16.82	>0.05	0.79 ± 0.2	19	>0.05	5.76 ± 1.16	15.11	>0.05	0.73 ± 0.11	12.39	>0.05
B3	6	8.3	8	0.29 ± 0.18	25.65	>0.05	0.86 ± 0.25	21.65	>0.05	6.24 ± 1.62	20.85	>0.05	0.77 ± 0.17	18.16	>0.05
B4	8	6.3	8	0.24 ± 0.1	16.22	>0.05	0.82 ± 0.21	19.08	>0.05	6.51 ± 1.34	16.99	>0.05	0.76 ± 0.1	11.01	>0.05
B5	10	5	8	0.49 ± 0.31	32.7	>0.05	1.11 ± 0.49	37.56	>0.05	6.82 ± 2.34	29.67	>0.05	0.95 ± 0.3	28.57	>0.05
SUM			40	0.26 ± 0.16	21.66		0.82 ± 0.22	23.59		5.89 ± 1.07	19.92		0.76 ± 0.12	17.92	

$P > 0.05$, difference is insignificant; $* 0.01 < P < 0.05$, difference is significant; $\dagger P < 0.01$, difference is very significant. Numbers of repeats were measured for each of the individual dilutions. SUM, subtotal.

FIGURE 1. Standard curves of four cytokines of one representative experiment. The standard curves of IL-1 β , IL-6, IFN- γ , and TNF- α were plotted by xPONENT 3.1 software (Luminex Corporation, Austin, TX) using the MFI against the concentrations of indicated analyte. The coefficient of variance, the R^2 for linearity regression, and minimal-maximal detection ranges given by xPONENT software are indicated. The ranges from 0.64 to 400 pg/mL points are boxed, and the points at 0.64 pg/mL are circled as reference points. The slope between MFI baseline and the log-linear phase line is also outlined.

Milliplex Assay Utilizing Pooled Tears. To ensure that tears can be analyzed with the same precision as commercial samples, the following experiments were performed.

Precision and Repeatability. Two pooled tear samples with large volumes (designated A and B) were independently collected from normal subjects and assayed. Ten microliters of tears from each was diluted into 40 μ L assay buffer to make a 5 $\times$ dilution. The dilutions were measured seven times (A) and eight times (B) as described above, and the mean $\pm$ SD of the concentrations was presented.

Effect of Tear Dilution. To evaluate the repeatability of tear dilutions, the two pooled tear samples (A and B) were each analyzed with 2, 4, 6, 8, or 10 μ L tears diluted to 50 μ L with assay buffer. This experiment had seven (A) or eight (B) replicates. The concentrations of each dilution are presented as the mean $\pm$ SD.

Effect of Tears on Assay Matrix: Spike Recovery Assay of Low, Middle, and High Levels of Standards. To determine if tears interfere with Luminex assay results, a spike recovery experiment was conducted using groups of 50 μ L/well samples, each containing 5 μ L of one of the two pooled tear samples (designated C and D) and 45 μ L of assay buffer with cytokine standards at various concentrations (0, 8, 40, or 200 pg/mL, respectively). Samples (six replicates) with cytokine standards alone were included as controls. The percentage of recovery of the standards in each group was calculated as the ratio of the mean of observed concentration over the expected concentration of each analyte.

Availability of Needed Tear Volume per Subject. To determine the ability to obtain sufficient tear volume from clinical subjects (DED or non-DED) using the SOPs, tear samples from right (OD) and left (OS) eye per subject were either collected separately (OD or OS tears) or pooled together (OD + OS tears) and their volumes determined.

Batch-to-Batch Repeatability. To create tear standards consistent over time and across batches, two pooled tear

samples, designated QC3A and QC3B, were collected and stocks of 10- μ L aliquots produced. Aliquots of each were measured twice, and the percent recovery and %CV were calculated, as described for the QC1 and QC2 standards, to yield the actual concentrations for batch-to-batch comparison. The unused aliquots, sufficient for 2 years of use, were stored in a -80°C freezer. Care was taken to prevent tear standards from running out before completion of a trial. Should the trial be completed for more than 2 years, new aliquots of QC3A and QC3B must be made the same way described above before the current aliquots are used up or expired.

Ability to Collect and Analyze Tear Samples From a Distant Site. To determine the ability to teach a distant site to obtain, store, and ship tear samples to the Biomarker Laboratory and demonstrate the ability of the laboratory to receive, track, and analyze received samples for multicenter clinical trials, interaction with an out-of-state site was instituted. Teaching was accomplished by a Web-based presentation followed by conference calls to review SOP details and answer questions. A total of 40 tear samples, 20 from contact lens wearers and 20 from age-matched noncontact lens wearers, were collected under an IRB-approved study and shipped to and assayed at Mount Sinai according to our SOPs.

Ability to Incorporate Tear Collection and Cytokine Analysis Into a Double-Blind RCT. Tears pooled from the right (OD) and left (OS) eyes of each DED subject in an IRB- and FDA-approved double-blind RCT study of systemic omega (ω)3 treatment were stored and analyzed at baseline and after 3 months of treatment according to the SOPs described above.

Statistical Analysis

Student's *t*-test (parametric), Wilcoxon-Mann-Whitney rank-sum test (nonparametric), and one-way analysis of variance (one-way ANOVA) on SPSS (version 17; SPSS, Inc., Chicago, IL)

FIGURE 2. Tear dilution effect and minimal tear volume (MTV) determination. Two pooled tear samples (A and B) were each analyzed with 2, 4, 6, 8, or 10 μL tears brought up to 50 μL by the assay buffer; each dilution was measured in seven (A) or eight (B) repeats. The mean concentrations of four selected cytokines were plotted against the tear volumes used in assay. Error bars represent the standard deviations of the mean. Multiple Student's *t*-tests were performed to compare the statistical differences among dilutions; the results with *P* values < 0.05 are marked with asterisks.

were run. Parametric results were presented as mean $\pm$ SD. Two-tailed significance was established at a confidence level of $0.05 > P > 0.95$.

RESULTS

MilliPlex Assay Utilizing Commercial Standards

Independent experiments were run with serial dilutions of standards and known concentration controls, each with duplicate measurements. Typical standard curves are shown in Figure 1. The intra-%CV is less than 1.7% and R^2 is approximately 1, making the “data fit” almost linear. Although the slopes between the initial baseline and an arbitrary linearity line matching the early to middle logarithm phase part of the curves were nearly the same for all four measured cytokines, the curve of IFN- γ displayed a bigger lag phase, which increased the maximum detecting capacity over 2000 pg/mL.

Precision, Accuracy, and Sensitivity. As shown in Table 1, a broad detectable range was achieved for each cytokine. The measured concentrations were almost the same as those of the standards ($R^2 \geq 0.99$). The inter-%CV of the four experiments was 15.86 for IL-1 β , 20.12 for IL-6, 40.04 for IFN- γ , and 39.47 for TNF- α . The MinDCs of the four experiments were 0.13 for IL-1 β , 1.22 for IL-6, 1.21 for IFN- γ , and 0.10 (pg/mL) for TNF- α .

Effect of Dilution of Standards on Accuracy. The mean of cytokine concentrations, the standard deviation of the mean, and the percentage recoveries are shown in Table 2. The recoveries at each diluting level over eight replicates were in the 80% to 120% range, although a slightly larger variance was seen at the lowest point (0.13 pg/mL), attesting to the approach toward the minimal detectable limit. The linearity of measured concentrations of all cytokines across dilutions was a near-perfect fit to the expected values of the standards ($R^2 > 0.99$).

Batch-to-Batch Repeatability. The measured mean concentrations of IL-6 and IFN- γ , both QC1 and QC2, were within the expected ranges (see Table 3), indicating 100% recovery. The mean recovery values of TNF- γ and IL-1 β in QC1 were also within the expected ranges, while those in QC2 were slightly above the upper ranges (105% for TNF- α and 129% for IL-1 β).

The %CVs were no more than 11%. These results met the quality control criteria set by the manufacturer.

MilliPlex Assay Utilizing Pooled Tears

Precision and Repeatability. With the pooled tear sample A, the %CVs of the four assayed cytokines were shown to be less than 20% at most of the dilution points. The mean %CVs of all the 36 measures across dilutions were also less than 20% (see Table 4). The concentrations were 0.11 ± 0.03 for IL-1 β , 0.56 ± 0.14 for IL-6, 4.49 ± 0.74 for IFN- γ , and 0.58 ± 0.07 pg/mL TNF- α . When 4 to 10 μL tears was measured, the *P* values were larger than 0.05 for all cytokines, indicating no statistical difference. Similar results were seen with pooled tear sample B.

Effect of Tear Dilution. A preliminary experiment using 2.5 to 10 μL of a pooled tear sample generated similar results ($P > 0.05$). This range is similar to the tear volumes usually obtained from DED patients. We therefore focused on two additional pooled tear groups (A and B) with more repeats (eight [A], seven [B]) to further narrow down the proper dilution ranges (5- to 25-fold). The mean concentrations displayed no statistically significant difference across the dilutions with tear volumes from 4 to 10 μL ($P > 0.05$), but

TABLE 5. Tear Volume Measurements

	Mean, μL	SD	N	% With Tear Volume $\geq 4 \mu\text{L}$
DED subjects				
OD or OS tears	2.64	1.11	29	14
OD + OS tears	14.18	11.95	92	81
Subtotal			121	
Non-DED subjects				
OD or OS tears	4	2.19	44	50
OD + OS tears	11.51	6.43	28	93
Subtotal			72	

Tears of the right (OD) or left (OS) eye of a subject were collected and measured as separate samples; OD + OS tears: tears of the left or right eye of a subject were collected and measured as one pooled sample.

TABLE 6. Spike Recovery With Tears and Cytokine Standards

Assays				IFN- γ			IL-1 β			IL-6			TNF- α		
Group	Tears, ID	Standards, μ L	Spiked Standards, pg/mL	Mean, pg/mL	SD	% Recovery	Mean, pg/mL	SD	% Recovery	Mean, pg/mL	SD	% Recovery	Mean, pg/mL	SD	% Recovery
Standards alone, $n = 6$	S0	0	0	<Threshold	NA	NA	<Threshold	NA	NA	<Threshold	NA	NA	<Threshold	NA	NA
	S1	0	8	8.21	5.8	103	8.24	2.42	103	7.97	3.49	100	7.64	3.11	95
	S2	0	40	48.58	16.97	121	39.68	11.28	99	34.91	15.61	87	33.51	15.48	84
	S3	0	200	196.48	37.91	98	233.81	38.59	117	171.86	46.02	86	240.9	130.44	120
Standard + tear-C, $n = 6$	S0-C	5	0	18.63	5.18	NA	0.64	0.28	NA	3.87	1.44	NA	1.54	0.33	NA
	S1-C	5	8	18.84	3.78	71	4.69	0.62	54	6.84	1.08	58	2.47	0.17	26
	S2-C	5	40	26.46	8.11	45	18.4	3.76	41	20.01	7.39	43	6.39	0.41	15
	S3-C	5	200	95.94	17.86	42	94.68	19.06	43	83.61	22.91	38	38.41	0.34	19
Standard + tear-D, $n = 6$	S0-D	5	0	12.83	5.31	NA	0.41	0.14	NA	2.89	0.62	NA	1.24	0.25	NA
	S1-D	5	8	14.72	5.16	71	5.25	1.5	63	6.73	2.23	62	2.3	0.25	25
	S2-D	5	40	28.51	11.03	52	18.02	3.61	40	18.5	5.91	40	6.2	0.34	15
	S3-D	5	200	101.22	20.38	44	89.97	21.15	41	92.58	34.14	42	40.9	0.4	20

The mean and SD were calculated from six replicate measurements in a 96-well plate assay. A single 50- μ L sample contains cytokine standards at concentrations of 0, 8, 40, or 200 pg/mL in assay buffer, in the presence or absence of 5 μ L tears. Two additional pooled tear samples (C and D) were collected and used in the experiment as indicated.

showed significant differences from those with 2 μ L (dilution factor of 25).

Upon normalization, the mean concentrations of all cytokines were plotted against tear volumes semilogarithmically, resulting in a nearly straight line with ≈ 0 slope and reasonable SD values (except for 2 μ L points; see Fig. 2). Thus, the minimal tear volume required for accuracy is greater than 2 μ L and, per our protocol, 4 μ L would be optimal.

Availability of Needed Tear Volume per Subject. To test the ability of collecting sufficient tears for analysis, a total of 193 tear samples were measured (121 samples from DED subjects, 72 from non-DED; see Table 5).

Among the DED tears, 29 samples were separately collected and measured from either right (OD) or left (OS) eyes ("OD or OS tears"). The mean tear volume $\pm$ SD was 2.64 ± 1.11 μ L (range, 0–5 μ L); only 14% of the samples had tear volumes ≥ 4 μ L. However, when OD and OS tears were pooled together ("OD + OS tears"), the mean $\pm$ SD went up to 14.18 ± 11.95 μ L ($n = 92$; range, 0–50 μ L); over 81% samples had tear volumes ≥ 4 μ L.

Among the non-DED tears, both the nonpooled and pooled OD + OS tears had volumes ≥ 4.0 μ L (see Table 5).

A pooled OD + OS tear sample, from either DED or non-DED subjects, would therefore likely ($\geq 81\%$) be sufficient for analysis utilizing the MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel (EMD Millipore Corporation). Since higher concentrations are expected in patients with inflammatory diseases, even smaller tear volumes might be accurately measured.

Batch-to-Batch Repeatability. The mean percent recovery of QC3A or QC3B was within the 80% to 120% range (see Table 3), and the mean %CVs were $\approx 20\%$.

Effect of Tears on Assay Matrix: Spike Recovery Assay of Low, Middle, and High Levels of Standards in Tears. In "Standards alone" groups (S0–S3) of the spike recovery results (Table 6), an overall 99% recovery was achieved (range, 84%–121%), similar to published results.^{35,40} In "Tears alone" groups (S0-C, S0-D), batches of pooled tears were similar in all cytokines, while in "Standards + Tear-C" or "Standards + Tear-D" groups (S1-C, S2-C, S3-C, S1-D, S2-D, S3-D), the overall recovery was $\approx 40\%$ (range, 15%–71%), with a trend toward decreasing recovery as the standards' concentrations increased.

Ability to Collect and Analyze Tear Samples From a Distant Site

There was no significant difference in tear volumes between contact lens wearers and nonwearers, or between the volumes reported by the collectors and those measured in the lab; there was no evaporation loss during tear transfer and storage.

All samples were received in good condition and sufficient volumes for analysis. The concentrations of inflammatory cytokines IL-6, IFN- γ , and TNF- α were not significantly different ($P > 0.05$) between the groups, and those of IL-1 β were only slightly higher in the contact lens wearer group ($0.05 > P > 0.01$; see Table 7).

Ability to Incorporate Tear Collection and Cytokine Analysis Into a Double-Blind RCT

To demonstrate the ability to incorporate tear sampling and cytokine analysis into a double-blind RCT, a feasibility double-blind RCT of $\omega 3$ for treatment of DED was performed utilizing

TABLE 7. Tear Cytokine Concentrations of Samples From Distant Site (pg/mL)

Cytokine	Normal		Contact Lens Wearers		P Value
	Mean $\pm$ SD	N	Mean $\pm$ SD	N	
IL-1 β	7.42 $\pm$ 5.62	20	15.56 $\pm$ 12.94	20	0.016*
IL-6	13.43 $\pm$ 8.74	20	14.86 $\pm$ 0.19	20	0.474
IFN- γ	0.27 $\pm$ 0.88	20	1.25 $\pm$ 3.69	20	0.261
TNF- α	7.46 $\pm$ 8.74	20	14.82 $\pm$ 15.8	20	0.078

For each subject, tears from right (OD) eye or left (OS) eye were collected and measured separately. *N* stands for the numbers of samples tested. Sample values below background were assigned as half of the minimal detection level of the cytokines, 0.07 for IFN- γ and 0.01 for TNF- α . Data are shown as mean $\pm$ SD. Paired two-tailed Student's *t*-test was run. Except for IL-1 β (*), which indicates IL-1 β level is considered statistically different between groups of normals and contact lens wearers, all the cytokines exhibited a *P* value > 0.05 between normal and contact lens wearers, and their differences were considered insignificant.

FIGURE 3. Tear cytokine levels in DED patients before and after 3 months of anti-inflammatory drug treatment. Tears pooled from the left (OS) and right (OD) eye of each DED subject in an IRB- and FDA-approved double-blind RCT study of systemic $\omega 3$ treatments were analyzed per SOPs. Patients received either $\omega 3$ or placebo (plcb) at randomized base. The concentrations of four selected cytokines were subsequently evaluated at baseline (pre-plcb or pre- $\omega 3$) and after 3 months of treatment (post-plcb or post- $\omega 3$). Changes of cytokine concentrations were calculated as shown on top of each figure. Trend of changes is highlighted by red arrows (with changes) and green arrows (no changes). The numbers (n) of patients per treatment groups are indicated.

the SOPs. There were no significant tear volume changes between the baseline and 3-month collections.

Tear samples were analyzed for cytokine concentrations in a masked fashion. Cytokine results were analyzed based on treatment after all patients completed the protocol and their data were entered into the relational database. The levels in three out of four selected tear cytokines displayed a tendency to decrease upon $\omega 3$ treatment: IL-1 β (4 $\times$ ↓), IFN- γ (3 $\times$ ↓), TNF- α (4 $\times$ ↓). Interleukin-6 was increased (4 $\times$ ↑) as compared to values in placebo subjects (Fig. 3; for details see Supplementary Table S3). These preliminary results suggest that $\omega 3$ may decrease inflammatory cytokines or delay progression of DED inflammation as compared to that in placebo controls; likely larger subject populations and longer treatment would be needed to determine efficacy of $\omega 3$ on reducing tear cytokine concentrations.

DISCUSSION

Clinical trials of DED often provide less than clear determination of efficacy, likely due to outcomes blurred by sources of bias in outcome measures, such as observer rating of corneal staining. Inclusion of minimally invasive objective metrics such as tear inflammatory cytokine profiles will aid in developing and determining the efficiency of new treatments as well as understanding changes in the ocular surface for specific external diseases, such as DED. The research reported here validates the SOPs needed to include tear sampling and reliable cytokine analysis in multicenter clinical trials.

To reliably include tear profiles as an ocular surface inflammatory biomarker, we first determined the sensitivities

and reliabilities of a MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel (EMD Millipore Corporation) for tear cytokine analysis. We evaluated the precision, repeatability, accuracy, sensitivity, effect of dilution, and batch-to-batch repeatability of the MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel (EMD Millipore Corporation) using the commercial standards, then confirmed the ability of the kit to process cytokine analysis of tears using the SOPs with pooled tears. Given that DED is likely to have multifactorial pathogenesis, simultaneous analysis of several key cytokines is essential. Given that tear samples, especially those obtained from individuals with DED, are likely small in volume and often nonrepeatable in assay, a highly sensitive methodology with reliable results is essential. Although methods such as ELISA^{60–62} and proteomic arrays^{60,63} have been used for multiple cytokine quantification, both require relatively larger sample volumes and are less sensitive than Luminex. Some recently introduced super high-sensitivity-high-throughput technologies, such as nanofibril-based nanoarray,^{64,65} are still under development. Our results demonstrated that the MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel (EMD Millipore Corporation) meets all the above requirements and is appropriate for the SOPs.

We then demonstrated that the profile of a panel of inflammatory cytokines (IL-1 β , IL-6, IFN- γ , and TNF- α) can be reliably used as a biomarker of DED. Extensive review of literature revealed that these four cytokines are the most frequently detected as increased in tears of DED subjects (Supplementary Tables S1, S2),^{14–16,22,32,33,37} play key roles in DED pathogenesis, correlate with mechanisms of immune modulation (such as IFN- γ increases in Th1 and IL-6 increases in Th17), and can be fit into one assay for simultaneous

quantification with the highest sensitivity. Although the SOPs were optimized with only four cytokines, the chosen panel could be expanded and tailored to fit any specific patient group or disease type.

Tear matrices are known to contain various substances that may interfere with Luminex assays. The absolute concentration of these substances and how they influence the accuracy of tear cytokine measurements may vary. Our spike recovery results in Table 6 indicate that, with just 5 μ L of tears, recovery results were significantly decreased. Similar interference was also observed by other peers using Luminex assay with tear or serum samples, or even with cytokine standards, probably due to multiple factors such as cytokine structures, and/or those of beads and sample matrix that could change the microreaction environment.^{35,40,58,66} Our results imply that the interfering effects vary: The most were seen with TNF- α and the least with IFN- γ . The interfering effects are not overcome by simply increasing concentrations of the standards, suggesting that factors other than inhibitors may be also involved, which is likely common in Luminex beads assay with many biological fluids.^{35,40,58,66} Therefore, a “specialized” buffering system designed specifically to work for a particular sample type such as a tear sample, and a “prefiltering” step prior to Luminex assay to remove or reduce the interfering substances, would be helpful.

Furthermore, data from published reports that include both DED and non-DED results indicate that the mean concentrations of these four inflammatory cytokines in normal individuals are 39.0 ± 23.6 pg/mL for IL-1 β , 42.2 ± 23.6 pg/mL for IL-6, 24.0 ± 18.0 pg/mL for IFN- γ , and 58.3 ± 36.9 pg/mL for TNF- α (see Supplementary Table S2).^{14–16,22,32,33,37} Results shown in Figure 2 and Table 4 were highly reproducible with volumes equivalent to 4 to 10 μ L of normal stimulated tears using the MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel (EMD Millipore Corporation). Taking these data together, we estimate that 4 μ L of normal tears could provide reliable results for all for cytokines. To support this, volumes of 193 nonstimulated tear samples from DED and non-DED subjects were measured. Results indicated that the mean tear volume of DED subjects was below 4 μ L (2.64 ± 1.11 μ L, $n = 29$) when tears of the left or right eye per subject were collected and measured separately. However, the mean tear volume would go up over 10 μ L when a pooled tear sample of both eyes per subject was used (14.18 ± 11.95 μ L, $n = 92$). Clinically, at least 4 μ L pooled tears could be collected from 93% of normal individuals and 81% of DED subjects by the SOPs (see Table 5). Therefore, a pooled tear sample from an individual is likely to be sufficient for Milliplex analysis. It needs to be mentioned that the benchmark of 4 μ L was justified based on measurements of the pooled normal stimulated tears, in which the concentrations of the cytokines could be even lower than those reported in nonstimulated tears of non-DED controls (Supplementary Tables S1, S2).^{14–16,22,32,33,37} Since tear samples from DED subjects are expected to have higher concentrations of inflammatory cytokines than those from normals, an even higher percentage of samples from DED subjects could likely be reliably evaluated.

Finally, we demonstrated that the SOPs for tear cytokine analysis could be reasonably incorporated into multicenter, double-blind RCTs. To achieve this goal, all involved sites would need to have trained personnel who could reliably collect, label, store, and ship samples to the Biomarker Laboratory for processing. The latter would then have to demonstrate the ability to receive, track, and analyze the masked samples and store the results electronically to send to a coordinate center for analysis. We demonstrated that, using the SOPs, a distant clinic site with Web-based education could

FIGURE 4. Scheme of SOPs. Critical steps to ensure reliable results are as follows: (1) tear collection by trained personnel with careful attention to masked labeling; (2) proper shipping of samples; (3) proper storage and tracking of samples with backup; (4) analysis with controls utilizing the MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panel; (5) accurate recording of masked results; (6) secure data collecting and transferring to the coordinating center for unmasking, analysis, and correlation with other clinical and laboratory findings. Serious consideration should also be given to the following: (1) including all commercial standards and reference controls (QC1 and 2) as well as additional tear aliquots (QC 3) for use over the length of the clinical trial with reasonable replicates; (2) utilizing assay kits from the same product lot of the same manufacturer—if a trial continues over an extended period (years), new lots of all kits must be regularly compared and standardized to those previously used; (3) calibrating pipettes periodically and standardizing the tips, tubes, and liquid transfer methods; (4) keeping sample and data tracking/logging records on a regular basis at every step, ideally with several cross-checking and secured backup systems running simultaneously; (5) creating a monthly report to the coordinating center and all of the participating clinical sites to provide quality control metrics ensuring compliance with SOPs; (6) deidentifying data only by the authorized personnel from the coordinating center.

obtain good-quality tear samples for inflammatory cytokine quantification. We also successfully incorporated the SOPs into a double-blind RCT of DED and traced the trend in cytokine changes between baseline and after 3 months of ω 3 treatment.

We also incorporated into our SOPs the previously established information important for tear cytokine profiling: collection of tears at the same time of the day for each subject given our knowledge of diurnal rhythm variation in tear cytokine concentration³⁶; use of capillary tubes rather than Schirmer strips for tear collection; maximum time (30 minutes) to keep tears on ice after collection and before freezing to ensure stability of cytokines; and avoidance of long-term tear sample storage at -80°C (less than 2 years) and multiple frozen-thaw cycles (no more than 2 cycles).³⁵ Critical steps in the SOPs to ensure reliable results are detailed in Figure 4. The SOPs ensure that results are reliable and help to eliminate sources of variation and bias found in other outcome measures typically used in DED clinical trials.

In summary, we have established and validated SOPs for human tear inflammatory cytokine assessment for use in multicenter clinical trials and demonstrated that the profile of tear inflammatory cytokines IL-1 β , IL-6, IFN- γ , and TNF- α can be used as a biomarker to monitor inflammatory diseases of the ocular surface such as DED. Tear cytokine biomarkers may provide critical information to fill the gap between clinical treatment modalities and the mechanism(s) involved in DED pathogenesis and response to treatment.

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