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SHORT COMMUNICATION

Long-Term Measurements of Human Inflammatory Cytokines Reveal Complex Baseline Variations between Individuals

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Comprehensive characterization of the healthy human proteome baseline is essential for personalized medicine. Baseline data are necessary to understand the variation between individuals, as well as longitudinal variation within individuals. Many important protein biomarkers, such as cytokines, exist at extremely low or undetectable levels in the healthy state. This paper describes results from a 14-week study of healthy human subjects using ultrasensitive single-molecule array (Simoa) assays to measure both intra and intersubject variation of 15 cytokines. The results show a wide variation in the ranges of some cytokines between individuals and demonstrate that individual baseline values will be essential for predicting disease presence and progression. Although all of the studied cytokines demonstrated high temporal stability (or low intrasubject variation) over the entire study period, there were two distinct groups of cytokines that demonstrated either high (IL-8, IFN-γ, IL-2, IL-6, and IL-1β) or low (IL-15, TNF-α, IL-12 p70, IL-17A, GM-CSF, IL-12 p40, IL-10, IL-7, IL-1α, and IL-5) subject-to-subject variation. This work demonstrates that ultrasensitive assays are essential for characterizing human cytokines in healthy subjects. The results show that some cytokines vary by more than two orders of magnitude between individuals, making it an imperative to obtain individual baseline measurements if they are to play a role in health and disease diagnosis. *(Am J Pathol 2017, 187: 2620–2626; https://doi.org/10.1016/j.ajpath.2017.08.007)*

Cytokines are small proteins secreted by immune cells that facilitate interactions and communications between cells.[1] By controlling various processes, including protein expression, gene regulation, and cell proliferation, cytokines are key regulators of the immune system and are essential in modulating both the innate and adaptive immune responses.[2,3] Measuring these immune mediators has increasingly attracted attention because they provide information about immunological functions, pathogeneses over the course of a disease or infection, and immune responses to therapies and interventions.[4–6] However, many cytokines cannot be measured because they exist in serum at concentrations below the detection limits of conventional immunoassay methods, making it a challenge to fully characterize and apply physiological cytokine baselines.

Although some cytokines are present at picomolar (pmol/L) levels or above in body fluids, most are expressed at femtomolar (fmol/L) levels or even lower. Many cytokines, such as IL-6, have been found to be important biomarkers in various diseases,[7–9] but they can be detected only during the acute phase when their levels are high. Because the levels of cytokines are correlated to disease types and stages, ultrasensitive methods that enable cytokine profiling in healthy or asymptomatic/pre-disease stage subjects can be highly informative for health monitoring or

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early disease diagnosis. Investigating the natural fluctuations in cytokine levels over time in individual subjects may provide insights into the physiological baseline and may enable earlier disease diagnosis because small perturbations can be detected. Such individual baseline profiles may facilitate personalized medicine.[10]

The inter- and intraindividual baseline variations of cytokines in serum have not been extensively studied.[11] Due to the lack of sufficiently sensitive detection technology, reported quantitative measurement of cytokines in serum and plasma have been limited to a few cytokines that either have high physiological concentrations or increase to detectable levels during acute disease states.[12,13] The relatively low sensitivity of current detection methods, such as enzyme-linked immunosorbent assay (ELISA), precludes the measurement of many informative cytokines, which becomes a barrier to the widespread clinical application of cytokine-based disease diagnostics and treatment.

Single-molecule array (Simoa) assays, also called digital ELISAs, have been developed to address this limitation in protein detection sensitivity. By integrating the conventional bead-based ELISA with microwell-array technology, limits of detection (LOD) reach the attomolar (amol/L) to fmol/L range (up to 1000-fold more sensitive than ELISA), thus enabling the ultrasensitive detection of proteins, including cytokines.[14–16] Simoa technology offers a new platform to quantify protein concentrations at previously undetectable levels, and has been applied for detecting protein biomarkers in the early stage of diseases, such as dengue virus infection,[17] ricin intoxication,[15] prostate cancer,[14] botulinum intoxication,[18] Crohn's disease,[19] and neurological disorders.[20]

In this paper, the Simoa technology was used to monitor the long-term (14-week) baseline cytokine levels in healthy human serum samples for 15 cytokines, most of which have concentrations in the fmol/L range or lower (Figure 1). Cytokine levels both within and between healthy subjects were measured to characterize the variation in human baseline cytokine levels.

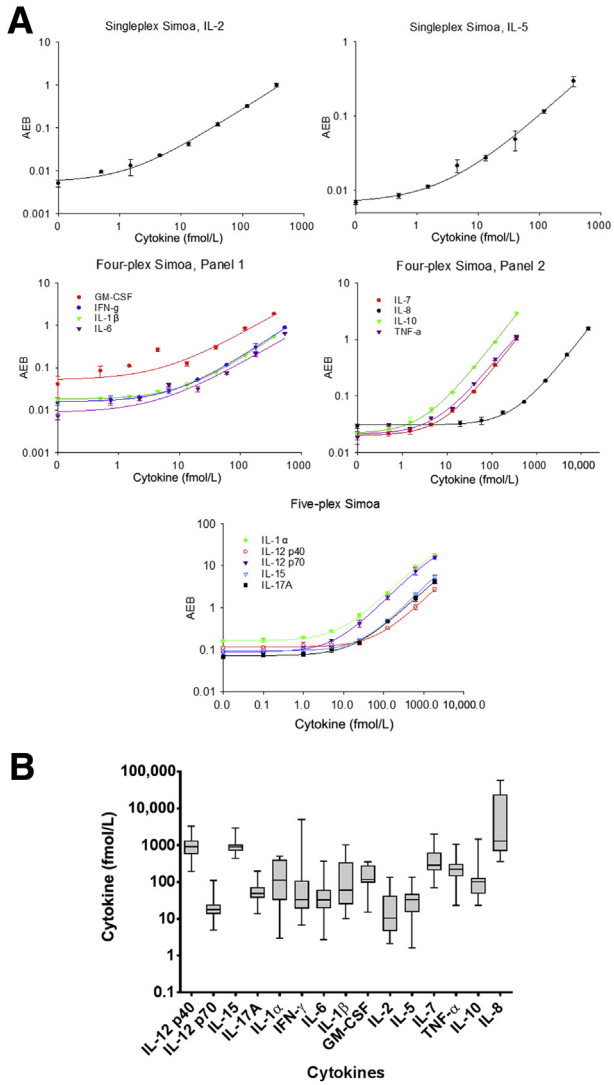

Figure 1 **A:** The log-log calibration curves for the detection of 15 cytokines in serum. There were five Simoa assays: two single-plex assays (IL-2 and IL-5), four-plex panel 1 (IL-6, IFN-γ, IL-1β, GM-CSF), four-plex panel 2 (IL-10, IL-7, TNF-α, IL-8), and a five-plex assay (IL-12 p40, IL-12 p70, IL-1α, IL-17A, IL-15). Experiments were performed in triplicate, and the error bars represent the standard deviations. Average enzymes per bead (AEB) are the standard unit for both digital and analog ranges in the Simoa assay. Data were analyzed by four-parameter logistic (4PL) curve fit ($1/y^2$ weighting). **B:** The human serum cytokine levels in all 89 samples of 10 subjects. The bar length indicates the interquartile range, and the inside segment indicates the median value. The whiskers above and below the box indicate the maximum and minimum values, respectively. Each sample was measured in triplicate. Only samples with detectable levels of cytokines were used in this analysis. The number of analyzed samples, that are detectable, for each cytokine are 89 samples for IL-12 p40, 61 samples for IL-12 p70, 89 samples for IL-15, 70 samples for IL-17A, 21 samples for IL-1α, 81 samples for IFN-γ, 87 samples for IL-6, 51 samples for IL-1β, 18 samples for GM-CSF, 83 samples for IL-2, 86 samples for IL-5, 89 samples for IL-7, 89 samples for TNF-α, 89 samples for IL-10, and 86 samples for IL-8.

Materials and Methods

Materials

The Simoa HD-1 Analyzer, five fluorescent dye-encoded multiplex bead types (488 L1, 647 L1, 647 L2, 700 L2, 700 L1) and the Simoa homebrew assay kit were purchased from Quanterix Inc. (Lexington, MA). The homebrew assay kit included carboxyl-functionalized paramagnetic beads (2.7 μm in diameter), streptavidin-β-galactosidase concentrate, resorufin β-D-galactopyranoside substrate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), diluents (bead diluent, detector diluent, and streptavidin-β-galactosidase diluent), buffers (bead wash buffer, bead conjugation buffer, bead blocking buffer, wash buffer 1, wash buffer 2), fluorocarbon oil, reagent bottles, pipette tips, 96-well microtiter plate, Simoa disks, and reaction cuvettes. Amicon Ultra-0.5 mL centrifugal filters 50 kD were purchased from EMD Millipore (Billerica, MA). Capture antibodies to granulocyte macrophage colony-stimulating factor

(GM-CSF), tumor necrosis factor-α (TNF-α), IL-1α, IL-2, IL-17A, IL-6, IL-12p40, and IL-15, and detection antibodies to GM-CSF, interferon-γ (IFN-γ), IL-1α, IL-2, IL-17A, IL-6, IL-15, and IL-5 were purchased from R&D Systems (Minneapolis, MN). Capture antibodies to IFN-γ, IL-1β, IL-7, IL-10, and IL-5, and detection antibodies to IL-1β, IL-7, IL-10, IL-12p40, and IL-12p70 were purchased from BioLegend (San Diego, CA). Capture and detection antibodies to IL-8 were purchased from BD Biosciences (San Jose, CA). Detection antibody to TNF-α was purchased from Abcam (Cambridge, MA). All of the human recombinant cytokines were purchased from R&D System with the exception of IL-1α, IL-17A, and IL-12p40, which were purchased from BioLegend. Phosphate buffered saline (PBS) and newborn calf serum were purchased from Life Technologies (Carlsbad, CA). ChromaLink biotin was purchased from Solulink Inc. (San Diego, CA). Zeba spin desalting columns, 7K MWCO, 0.5 mL, were purchased from Thermo Fisher Scientific (Waltham, MA).

Preparation of Capture Beads with Capture Antibodies

The multiplex beads were purchased with pre-encoded fluorescent dyes to generate multiple distinct bead populations. Detailed information about the dye-encoding process can be found elsewhere.[21] The antibody coupling onto the paramagnetic beads is similar for both beads with or without dye encoding. In brief, approximately 2.3×10^8 paramagnetic beads were washed three times with bead wash buffer and two times with bead conjugation buffer. The washed beads were suspended in 190 μL of bead conjugation buffer, followed by the addition of 10 μL of 10 mg/mL EDC solution. The mixture was incubated for 30 minutes on a plate shaker at 1000 rpm. Beads were washed once with bead conjugation buffer, followed by the addition of 200 μL of 0.5 mg/mL capture antibody in bead conjugation buffer. The mixture was incubated for 2 hours on a plate shaker at 1000 rpm. After washing two times with bead wash buffer, the beads were incubated with 200 μL of bead blocking buffer for 30 minutes on a plate shaker at 1000 rpm. The beads were washed once with bead wash buffer, once with bead diluent buffer, and were stored in 200 μL of bead diluent buffer at 4°C.

Preparation of Biotin-Conjugated Detection Antibodies

Detection antibodies of anti−GM-CSF, anti−TNF-α, anti−IFN-γ, and anti−IL-2, were biotin conjugated in house following the manufacturer's protocol (Solulink Inc.). In brief, a Zeba spin desalting column was washed three times with modification buffer (100 mmol/L sodium phosphate, 0.15 mol/L NaCl, pH = 8). Five hundred microliters of 1 mg/mL detection antibody was added to the washed column, and the column was centrifuged at 1500 × *g* for 2 minutes. Meanwhile, 100 mg/mL ChromaLink biotin was prepared in anhydrous dimethylformamide. Biotin solution was added to desalted antibody in eightfold to ninefold excess, and the mixture was incubated for 90 minutes at room temperature without shaking. While incubating, two new Zeba columns were washed with 1× PBS. After incubation, antibodies were purified by passing through each Zeba column at 1500 × *g* for 2 minutes. Purified biotin-conjugated detection antibodies were stored at −20°C. All other detection antibodies were purchased with biotin conjugates and were stored at the manufacturer's recommended storage conditions.

Simoa HD-1 Analyzer

All reagent solutions [5×10^6/mL capture antibody−coated paramagnetic beads, biotin-conjugated detection antibodies (see *Preparation of Biotin-Conjugated Detection Antibodies* for the detection antibody concentration), 150 pmol/L streptavidin-β-galactosidase reagent, 1× PBS buffer, 100 μmol/L resorufin β-D-galactopyranoside substrate, wash buffer 1, wash buffer 2, and fluorocarbon oil] were loaded into the instrument. Serial dilutions of the cytokine calibrators were prepared in 1× PBS containing 25% newborn calf serum. The calibrators and human serum samples were loaded into a 96-well plate and inserted into the sample bay. Human serum samples were fourfold diluted in 1× PBS buffer. Calibrators and samples were run in triplicate.

The human serum samples were screened for 15 cytokines using a total of five Simoa assays: two single-plex assays and three multiplex assays. Specifically, there were two single-plex assays (IL-2 and IL-5), two four-plex assays (IFN-γ, IL-6, IL-1β, GM-CSF; and IL-10, IL-7, TNF-α, IL-8), and one five-plex assay (IL-12 p40, IL-12 p70, IL-1α, IL-17A, IL-15). All reagent solutions were prepared in the same manner for all single-plex and multiplex assays, except for the antibody-coated beads solution. In a single-plex assay, the bead solution was prepared at 5×10^6 beads/mL. In a four-plex assay, the bead solution was prepared with 1.25×10^6 beads/mL for each bead type. In a five-plex assay, the bead solution was prepared with 1×10^6 beads/mL for each bead type. In each assay, the final bead concentration was 5×10^6 beads/mL.

After all assay materials were loaded into the instrument, the Simoa HD-1 analyzer performed the following steps for every calibrator and diluted sample: 100 μL of the bead solution was pipetted into a reaction cuvette and pelleted with a magnet, the supernatant was removed, and 100 μL of the calibrator or diluted serum sample was added. The mixture was incubated for 15 minutes. The beads were washed with wash buffer 1 and pelleted, the supernatant was removed, and the beads were then incubated with 100 μL of biotin-conjugated detection antibody solution for 5 minutes. The beads were washed with wash buffer 1 and pelleted, the supernatant was removed, and the beads were then incubated with 100 μL of streptavidin-β-galactosidase enzyme solution for 5 minutes. The beads were subsequently washed with wash buffer 1 and wash buffer 2, and were then suspended in

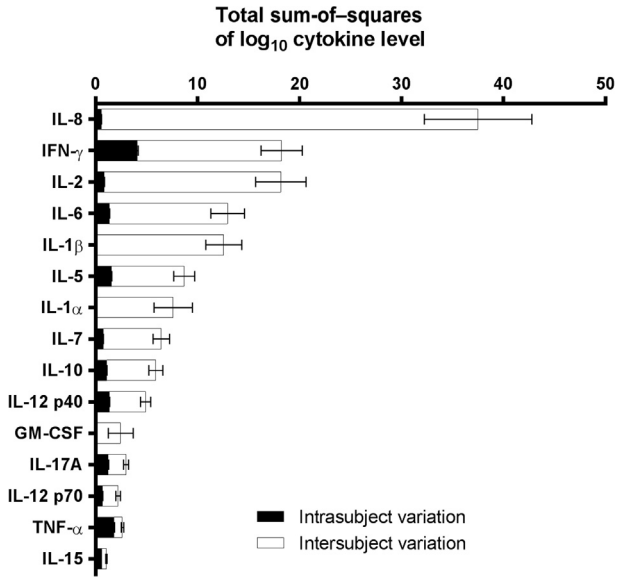

Figure 2 Variation in baseline cytokine levels in eight healthy subjects. Only samples with detectable levels of cytokines were used in this analysis. The length of the bar denotes the total variance (total sum-of—square), and the white and black designate the fraction of total variance attributed to intersubject and intrasubject variations, respectively. Error bars represent mean square error values of the variance. Number of analyzed samples, that are detectable, for each cytokine are 69 samples for IL-12 p40, 44 samples for IL-12 p70, 69 samples for IL-15, 52 samples for IL-17A, 20 samples for IL-1α, 62 samples for IFN-γ, 68 samples for IL-6, 41 samples for IL-1β, 17 samples for GM-CSF, 66 samples for IL-2, 69 samples for IL-5, 69 samples for IL-7, 69 samples for TNF-α, 69 samples for IL-10, and 69 samples for IL-8.

25 μL of resorufin β-D-galactopyranoside substrate solution. Following this, 15 μL of the bead—substrate solution was loaded via gravity into the 216,000 femtoliter-well array on a 24-array Simoa disk,[22] in which one well can fit only one bead. The wells were sealed with fluorocarbon oil. Only wells (*On* wells) with the enzyme-labeled immunocomplex beads hydrolyzed the resorufin β-D-galactopyranoside substrate to generate the fluorescent product resorufin.

Two resorufin-channel fluorescence images, excitation/emission of 574 nm/615 nm, of the well-array were taken at a 30-second interval, with a threshold of at least 20% increase in fluorescent intensity, to determine the number of On wells versus the random fluorescent background. Subsequent fluorescent images were taken at excitation/emission of 680 nm/720 nm for the identification of 700 L1 and 700 L1 bead types, 622 nm/667 nm for the identification of 647 L1 and 647 L2 bead types, and 490 nm/530 nm for the identification of 488 L1 bead type. Using these pre-identified fluorescent multiplex beads, the Simoa resorufin signal in On wells could be attributed to the respective cytokine subpopulations in the multiplex assays. A four-parameter logistical curve (4PLC, $1/y^2$ weighted) was applied for the average enzymes per bead as a function of cytokine concentration curve fitting.

Participant Recruitment, Sample Collection, and Preparation

Participant recruitment and sample collection followed a Tufts University Institutional Review Board—approved protocol (Study number 1410019). Informed consent was obtained from all participants in this study.

Ten asymptomatic participants, male and female, coded as P1 to P10, were recruited from Tufts University (18 to 35 years of age). At each study visit over the 14-week period, 10 mL of whole blood was collected via venipuncture from the participants' arm into vacutainer tubes.

Table 1 Specifications of 5 Simoa Assays for the Detection of 15 Cytokines

Cytokine	Assay LOD, fmol/L	Sample LOD, fmol/L	Detectable samples, % (n = 89)	LOD fold-improvement vs ELISA kits	Simoa assay-plex
IL-2	0.50	2.0	93	62	Single-plex
IL-5	0.26	1.1	97	523	Single-plex
IL-6	0.50	2.0	98	55	Four-plex panel 1
IFN-γ	2.3	9.3	92	30	Four-plex panel 1
IL-1β	1.6	6.4	57	4	Four-plex panel 1
GM-CSF	5.8	23	20	6	Four-plex panel 1
IL-10	0.38	1.5	100	53	Four-plex panel 2
IL-7	3.0	12	100	3	Four-plex panel 2
TNF-α	3.0	12	100	6	Four-plex panel 2
IL-8	76	3.0×10^2	97	1.1	Four-plex panel 2
IL-12 p40	5.7	23	100	40	Five-plex
IL-12 p70	0.94	3.8	69	17	Five-plex
IL-1α	0.60	2.4	24	185	Five-plex
IL-17A	2.8	11	80	78	Five-plex
IL-15	5.0	20	100	53	Five-plex

There were two single-plex assays (IL-2 and IL-5), two four-plex assays (IL-6, IFN-γ, IL-1 β, GM-CSF; and IL-10, IL-7, TNF-α, IL-8), and one five-plex assay (IL-12 p40, IL-12 p70, IL-1α, IL-17A, IL-15). Sample limit of detection (LOD) is equal to four times the assay LOD to account for fourfold sample dilution. Detectable samples are those with concentration values greater than the LOD. The fold-improvement was calculated using the most sensitive single-plex ELISA kits of four commercial vendors: R&D Systems, BioLegend, BD Bioscience, and Abcam, from which the antibodies were purchased.

Blood samples from all of the volunteers were intravenously collected between 5 and 7 PM on every Monday (or on Tuesday if Monday was a holiday or school closure day) during the 14-week study. Immediately following collection, blood samples were allowed to settle at room temperature for 1 hour in the vacutainer tubes. The tubes were then centrifuged at 1026 × *g* for 15 to 20 minutes to separate the serum layer. The serum samples were collected into a new tube and stored at −80°C until use. A total of 89 samples from 10 volunteers over a 14-week period were used in this study.

Over the course of the 14-week study, six volunteers (P1 to P5 and P7) had 10 blood draws, two volunteers (P6 and P8) had 9 blood draws, one volunteer (P9) had 6 blood draws, and one volunteer (P10) had 5 blood draws.

Quantification of Baseline Intra- and Intersubject Variations

Two individuals P1 and P3, self-reported as sick during the study and therefore were excluded from the healthy baseline profile. A total of 69 samples from 8 healthy individuals were used for this analysis.

To establish the baseline profiles in healthy human subjects, cytokine concentrations, $\log_{10}$ transformed, were fitted in the analysis of variance model (GraphPad Prism software version 7; GraphPad Software, La Jolla, CA), yielding the total variance (total R^2 sum of squares), the temporal stability (intrasubject), and the subject-to-subject variation (intersubject). The variation analysis was computed for 95% confidence. The total sum of squares is denoted by the length of the bar with intersubject variation and intrasubject variation shown in white and black, respectively (Figure 2). The mean square error values are shown as error bars in Figure 2 to denote the variance in the inter- and intrasubject variation analysis.

Results

Single-plex and multiplex Simoa cytokine assays with high sensitivity and specificity were developed for screening serum samples. The concentrations of the detection antibodies and the dynamic range for each assay were optimized to the physiological level of each cytokine in serum samples (*Materials and Methods*). The log-log calibration curves for these assays are shown in Figure 1. In total, 89

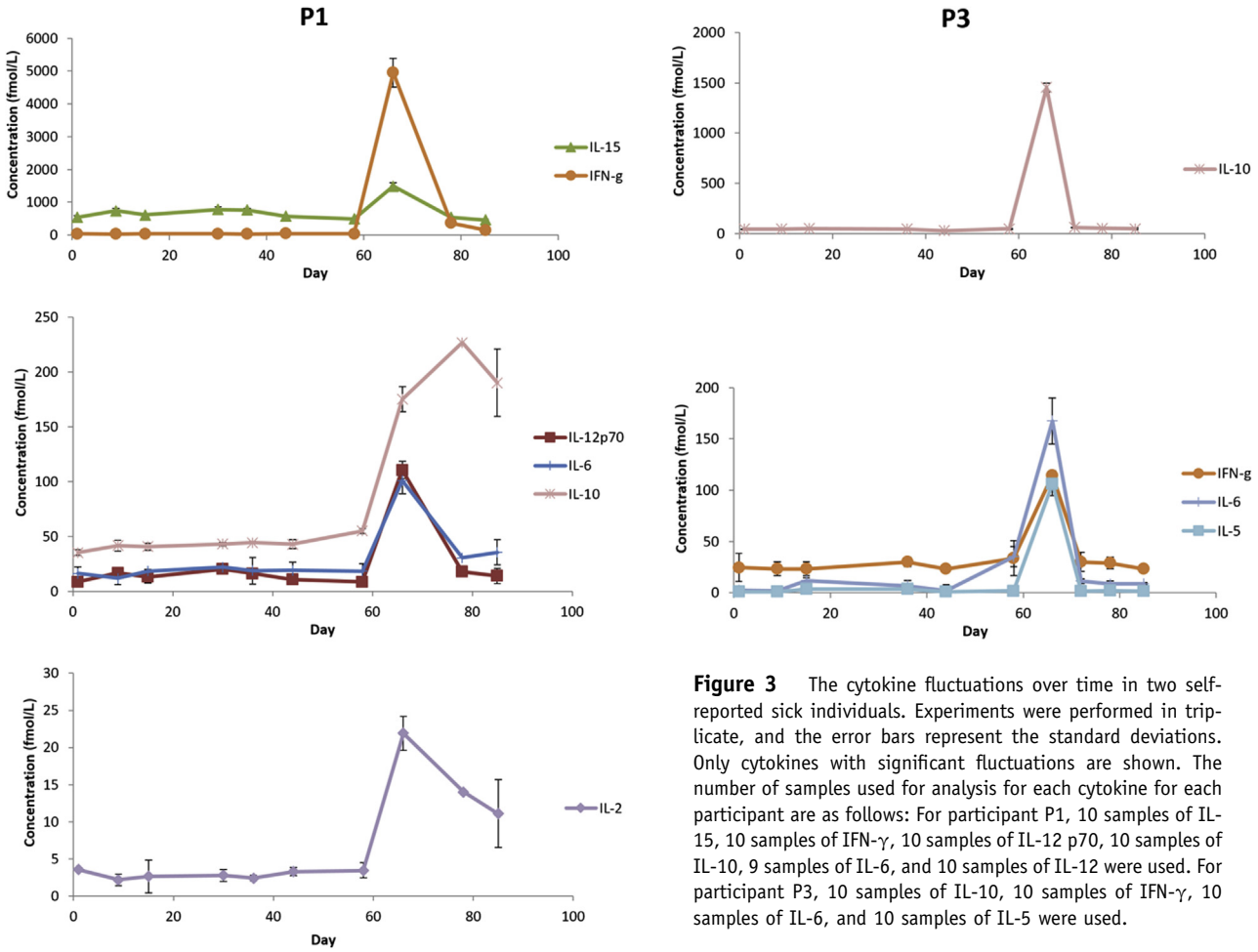

Figure 3 The cytokine fluctuations over time in two self-reported sick individuals. Experiments were performed in triplicate, and the error bars represent the standard deviations. Only cytokines with significant fluctuations are shown. The number of samples used for analysis for each cytokine for each participant are as follows: For participant P1, 10 samples of IL-15, 10 samples of IFN-γ, 10 samples of IL-12 p70, 10 samples of IL-10, 9 samples of IL-6, and 10 samples of IL-12 were used. For participant P3, 10 samples of IL-10, 10 samples of IFN-γ, 10 samples of IL-6, and 10 samples of IL-5 were used.

serum samples from 10 volunteers were screened. The detectable cytokine levels are shown in Figure 1 and Table 1. LODs were determined by adding three times the SD of the background to the background signal. It is important to note that because IL-8 has a comparatively high expression level (approximately 1 pmol/L) in healthy human samples, there was no need to develop an ultrasensitive assay. By reducing the detection antibody concentration, an IL-8 assay that avoided signal saturation was able to be developed and allowed the Simoa assay to be performed with all of the measured cytokines.

To establish the baseline profile of cytokines in healthy subjects, the temporal changes around the baseline within subjects (intrasubject variation) and the baseline differences between subjects (intersubject variation) were examined. For this baseline analysis, samples obtained from healthy subjects at all collection time points were used. Two individuals (P1 and P3) self-reported as sick during the study and were excluded from the baseline analysis. Therefore, a total of 69 samples from 8 individuals were used for the baseline analysis.

As shown in Figure 2, there is low intrasubject variation for all individuals. All cytokines demonstrate high temporal stability throughout the 14-week period. On the other hand, intersubject variability is high depending on the particular cytokine. Significantly, two distinct groups of cytokines were observed: one group had a low intergroup baseline variation, whereas the other group had high intergroup baseline variation. The first group, including IL-15, TNF-α, IL-12 p70, IL-17A, GM-CSF, IL-12 p40, IL-10, IL-7, IL-1α, and IL-5, showed low subject-to-subject variability, whereas the second group, including IL-8, IFN-γ, IL-2, IL-6, and IL-1β, showed substantial subject-to-subject variability.

During this 14-week study, there were two individuals who self-reported as sick (P1 reported a fever of 102°F, sore throat for several days, swollen glands, and mild headache on day 44; P3 reported swollen glands and possible sinus infection on day 66). Although there were no professional clinical diagnoses available for any of these occurrences, some significant cytokine elevations above the baseline in these individuals were able to be observed (Figure 3).

Discussion

Multiplexing the 15 cytokines into three multiplex assay panels and two single-plex assays reduced the required sample volume for a triplicate measurement from 1125 to 375 μL (a 67% decrease), making the assay panel amenable to routine diagnostics. To calculate the fold improvement, Simoa LODs were compared to commercial ELISA kits from four vendors (R&D Systems, BioLegend, BD Bioscience, and Abcam), from which the antibodies were purchased (*Materials and Methods*). The Simoa assays had LODs in the range of 0.26 to 76 fmol/L, demonstrating up to 500-fold (an average of 74-fold) improvement over commercial ELISA kits. This enhanced sensitivity allowed the detection of cytokines in 82% of the serum samples compared to only 25% that would have been detectable using ELISA. Therefore, with Simoa, a significantly more complete analysis of these cytokines was obtained.

For cytokines in the low-variation group, one could use baseline values to establish a normal serum level range that would be representative of most individuals. Any significant deviation from this normal range could potentially be an indication of an immune response. Therefore, these cytokines have potential as valuable biomarkers for clinical detection of host immune responses, because a single measurement outside the normal range could be used to determine whether someone were ill or pre-symptomatic and about to get sick. However, in the high-variation group, because the baseline variations are substantial even between healthy individuals, a long-term personalized baseline profile would be required to detect abnormal cytokine levels. In this cohort, all of the subjects were healthy college students, yet some of their baseline cytokine levels differed by a factor of hundreds to thousands. One possibility is some of these individuals may be poised to be either more or less susceptible to infection. Without additional studies, it is not possible to determine how such large differences in baseline levels of some cytokines affect human health. It is important to note that IL-1α and GM-CSF have low detectability percentages, 24% and 20%, respectively. Therefore, the variation calculations for these two cytokines should be viewed as statistically less significant in comparison to other cytokines. The inability to detect these cytokines is specific to particular donors, indicating their baseline levels were low. By contrast, all samples for P2 and P10 donors were detectable for IL-1α and GM-CSF, whereas samples of other donors (P1 and P3 to P9) were mostly undetectable for these cytokines.

Overall, the cytokine patterns varied among all individuals. Although these observations are insufficient to draw any definitive conclusions, the significant excursions in cytokine levels suggest that early detection of disease due to the host response may be possible if baseline measurements are obtained. Although the variations observed in this study were obtained with only 69 samples, the stability of the individual cytokines within a subject over 14 weeks and the significant variation in some cytokines between subjects suggest that the results are robust. A more comprehensive study with detailed clinical information, diagnoses, and larger sample size is required to provide further insights into the correlation of these cytokine fluctuations with types and stages of infection or illness.

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D.W. and T.L.D. contributed equally; D.W., T.L.D., and D.R.W. conceived the project; D.W. and T.L.D. designed the institutional review board–approved sample collection and the experiments; D.W., T.L.D., and B.P.B. performed the experiments and interpreted the results; D.W. and T.L.D. wrote the manuscript with significant input from B.P.B. and D.R.W; all authors have given approval to the final version of the manuscript.

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