

Reproducibility of LPS-Induced ex vivo Cytokine Response of Healthy Volunteers Using a Whole Blood Assay

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Introduction: Lipopolysaccharide (LPS) stimulation of human whole blood ex vivo has been widely used to investigate human innate immune responses. However, there are uncertainties regarding the reproducibility and reliability of this assay.

Methods: In this prospective, single-center study, cytokine responses (interleukin 8, interferon- α , interferon- γ , interleukin 10, interleukin 1- β , interleukin 6, and tumor necrosis factor- α) to ex vivo whole blood LPS stimulation were assessed in 12 healthy volunteers. Cytokine levels were measured at 0, 2, and 4 h using a multiplex immunoassay (Luminex[®]). Stimulation was repeated after six weeks. We examined reproducibility across technical and biological replicates at baseline and between repeated experiments after 6 weeks based on the area under the curve (AUC) of the individual cytokines using Pearson's correlation coefficient and the mean coefficient of variation.

Results: The lowest mean coefficients of variation were observed for the technical replicates (5.4 to 9.2%), followed by the biological replicates (8.1 to 24.8%), and the repeated experiments after 6 weeks (17 to 31.2%). Between the baseline and 6-week AUCs, the following Pearson correlation coefficients R were observed: interleukin 10, 0.97; interferon- α , 0.84; interleukin 1- β , 0.83; interleukin 8, 0.79; interleukin 6, 0.73; interferon- γ , 0.73; and tumor necrosis factor- α , 0.63.

Discussion: The level of agreement between the baseline and week-6 cytokine response to ex vivo LPS stimulation was high across the seven cytokines analyzed. While interleukin 10 exhibited the lowest level of variability over time, tumor necrosis factor- α showed the highest variability in repeated experiments, which should be considered in the design and interpretation of future studies.

Keywords: LPS, lipopolysaccharide, inflammation, endotoxin

Introduction

The ex vivo whole blood lipopolysaccharide (LPS) stimulation assay has been widely used to explore individual cytokine reactivity as part of the human innate immune responses.¹ Moreover, the ex vivo cytokine response to LPS has been shown to predict outcomes in various clinical situations. Hall et al demonstrated that immunoparalysis, as determined by an inadequate ex vivo whole blood response to LPS, is associated with increased mortality in critically ill patients.² Similarly, Ploder et al found that a reduced ex vivo LPS-induced TNF- α production serves as an early predictor of adverse clinical outcomes in trauma patients with sepsis.³

However, uncertainty exists regarding the long-term stability of ex vivo cytokine responses to LPS. In particular, it is unclear to what degree the observed cytokine production represents a stable personal trait (mainly determined by genetics and sex) or is influenced by time-varying immunological factors, such as stress, disease exacerbations, seasonality, or

infections. Previous studies investigating the stability of the cytokine response to LPS in the ex vivo whole blood assay found conflicting results and examined only a small number of relevant cytokines.⁴⁻⁶

Our study aimed to extend this area of research by investigating additional cytokines and exploring analytical sources of variability. We assessed ex vivo whole blood LPS induced cytokine responses in healthy volunteers at baseline and after a 6-week interval. We also examined the variation in technical and biological replicates to provide a more nuanced understanding of the assays' reliability.

By investigating these aspects, our study aimed to elucidate the uncertainty regarding the methodological robustness of ex vivo whole blood LPS challenge. These results may provide novel insights for the design and interpretation of research aimed at exploring the complexities of innate immunity using the ex vivo whole blood LPS challenge.

Methods

Study Design and Participants

This prospective, single-center study included six female and six male healthy volunteers to perform an ex vivo whole blood LPS stimulation at baseline and after 6 weeks. The key inclusion criteria were physical and mental health and age ranging from 20 to 30 years. The key exclusion criteria were smoking, history of substance abuse, use of medication other than birth control, weight >95 kg, weight <60 kg for men or <50 kg for women, any vaccination within 4 weeks before the first study day, and previous participation in an intravenous LPS study. The exact inclusion and exclusion criteria are presented in [Supplementary Table 1](#).

This study was conducted in accordance with the principles of Good Clinical Practice and the Declaration of Helsinki. This study was approved by the Ethics Committee of the Medical University of Vienna (ID 1676/2020). All subjects provided oral and written consent to participate in the study.

Ex vivo Whole Blood Stimulation

Blood sampling for the ex vivo stimulation was scheduled between 8 h and 9 h in the morning of study days. [Supplementary Table 2](#) lists the lifestyle restrictions that ensured standardized conditions before stimulation. Blood collection was standardized to minimize preanalytical errors. Extensive use of tourniquets, undersized needles, excessive shaking of tubes, and vein tapping were avoided to prevent hemolysis and mechanical stress to the blood sample. Blood was collected in five 9 mL heparin-coated tubes. Each tube was gently inverted 8 to 10 times to ensure adequate anticoagulation of the samples. The baseline samples were immediately centrifuged and processed. The 2-hour sample was incubated at 37°C for two hours before it was spiked with LPS and incubated for another two hours. The 4-hour sample was immediately incubated with LPS for 4 hours. Each sample contained 9 mL of whole blood that was stimulated with 1 mL of an LPS (*E. coli* O:113 Reference Endotoxin, CC-RE Lot 3) solution at 500 pg/mL to achieve a final concentration of 50 pg/mL, which can be achieved in human endotoxin challenge models^{7,8} and which is a representative value that is reached in the plasma of septic patients.^{9,10} During the 4-hour incubation, all sample tubes were placed horizontally on a shaking plate with a shaking frequency of 10 per minute. After incubation, the stimulated whole blood was centrifuged at 4°C and 500 × g, and the supernatant was stored at -80°C in aliquots for cytokine measurements. In seven of the 12 subjects, we performed two biological replicates of the 2h and 4h sample at baseline (simultaneous LPS stimulation in two separate tubes).

Cytokine Quantification with Luminex

The Luminex[®] Multiplex immunoassay is a magnetic microparticle-based immunoassay that uses the same sandwich principles as traditional ELISA.¹¹ In brief, magnetic beads, each with a characteristic fluorescence pattern for discrimination and each pre-coated with its own analyte-specific antibody, were added to the samples. After the addition of biotinylated detection antibodies and phycoerythrin (PE)-conjugated streptavidin, the beads were read on a dual-laser flow-based detection instrument. Measurements were performed in two separate wells per sample (technical replicates). A specific kit containing antibodies for the following seven analytes was used: interleukin 1-β, interleukin 6, interleukin 8, interleukin 10, TNF-α, interferon-α, and interferon-γ. These seven cytokines are known to be released following LPS

stimulation.¹² The lower limits of quantification were as follows: interleukin 10, 36.2 pg/mL; interferon- α , 4.7 pg/mL; interleukin 1- β , 3.4 pg/mL; interleukin 8, 1.5 pg/mL; interleukin 6, 9.5 pg/mL; interferon- γ , 8.1 pg/mL; and TNF- α , 7.3 pg/mL. [Supplementary Table 3](#) shows the performance of the repeated measurement of the low and high concentration quality control (QC) samples. The low concentration QC samples had coefficients of variation below 15%, except for interleukin 1- β , which showed a coefficient of variation of 17.0%. All high concentration QC samples showed a coefficient of variation below 10%. [Supplementary Figure 1](#) shows the linearity of the standard curves for the individual cytokines.

Data Analysis

Baseline characteristics were reported descriptively for male and female subjects using mean \pm standard deviation (SD) and number (%). Ex vivo cytokine responses were analyzed based on the area under the curve from baseline to 4 hours (AUC_{0-4}) for each cytokine using the trapezoidal formula.

The primary endpoint of this study was the reproducibility of the ex vivo cytokine response to LPS. To this end, we assessed the correlation of the individual cytokine AUCs between the technical replicates, biological replicates, and repeated experiments after six weeks using the Pearson correlation coefficient. As previously suggested, Pearson correlation coefficients $R < 0.4$ indicate low, 0.4 to 0.7 moderate, 0.7 to 0.9 high, and > 0.9 very high correlation.¹³ For this exploratory study, the sample size was fixed based on the strengths of the correlations that could be detected. Considering an α of 0.05, a sample size of 12 would have 85% power to detect a Pearson correlation coefficient of $R = 0.75$. In addition, we visualized the respective levels of agreement using Bland-Altman plots.¹⁴ The mean coefficient of variation (%) was calculated for each cytokine AUC for the technical replicates, biological replicates, and repeated experiments. The coefficient of variation (CV) was calculated using the following formula: $CV = (SD/Mean) \times 100$. For the repeated samples, we performed an additional analysis, in which we normalized the cytokine AUCs at week 6 according to the ratio of leukocytes in the white blood count at baseline and week 6. In an exploratory analysis, we compared the cytokine response at baseline between male and female subjects and between subjects with or without a history of Covid-19 using an independent sample *t*-test. Among subjects who had Covid-19, we assessed the correlation between the time since symptomatic Covid-19 (in days) and the response of the individual cytokines at baseline. We also evaluated the correlation between individual cytokine levels and previous Covid-19 vaccinations or the latest immunological events (ie, SARS-CoV-2 infection or vaccination, whichever occurred later). The association between seasonality and cytokine response was assessed visually. The fitted line and error range were plotted using the locally estimated scatterplot smoothing (LOESS) method of the `geom_smooth` function of the `ggplot2` package. No statistical inferential model for seasonality was run due to the limited number of observations. Statistical analysis and visualization were performed using R (version 4.1.2, 2021, Vienna, Austria) and RStudio (RStudio Team, 2020).

Results

Participants

A total of 12 participants (6 males and 6 females) were included in this study. The baseline characteristics are shown in [Table 1](#). The mean \pm SD age was 24.2 ± 2.9 years. As expected, males had a greater body height (184 ± 7 vs 168 ± 7 cm) and weight (77 ± 11 vs 64 ± 11 kg), but a similar body mass index (23 ± 3 vs 23 ± 4 kg/m²). The mean time from baseline to follow-up ex vivo LPS stimulation was 44 ± 5 days (that is 6.3 ± 0.7 weeks). A previous episode of symptomatic Covid-19 was reported by 8 (75%) of the 12 participants. All subjects (100%) received at least one SARS-CoV-2 vaccine prior to the study. Ten subjects received a booster vaccination (third shot) with the Pfizer-BioNTech mRNA vaccine. Two subjects received the primary series (two shots) of Pfizer-BioNTech vaccine.

Overview of Findings

[Table 2](#) lists the mean coefficients of variation for each cytokine for the technical replicates, biological replicates, and repeated experiments after six weeks. The lowest mean coefficients of variation were observed for technical replicates (5.4 to 9.2%), followed by biological replicates (8.1 to 24.8%). The highest mean coefficients of variation were observed

Table 1 Baseline Characteristics of Included Subjects

	Total	Females	Males
N=	12	6	6
Age, years (mean (SD))	24.2 (2.9)	22.7 (2.4)	25.7 (2.7)
Height, cm (mean (SD))	176.3 (10.7)	168.3 (6.9)	184.3 (7.1)
Weight, kg (mean (SD))	70.8 (12.7)	64.2 (11.1)	77.4 (11.2)
BMI, kg/m ² (mean (SD))	22.7 (3.2)	22.7 (3.8)	22.8 (2.8)
Any Allergies (n (%))	4 (33.3)	2 (33.3)	2 (33.3)
Previous Covid-19 (n (%))	8 (75)	5 (83.3)	3 (50)
Vaccinated against Covid-19 (n (%))	12 (100)	6 (100)	6 (100)
Oral contraception (n (%))	N/A	4 (66.7)	N/A

Table 2 Mean Coefficient of Variation (%) per Cytokine

Cytokine	Technical Replicates (n=12)	Biological Replicates (n=7)	Repeated Experiments (n=12)
Interleukin 8	7.8	16.4	23.0
Interferon- α	9.3	21.4	24.6
Interferon- γ	7.4	8.2	24.5
Interleukin 10	7.7	13.7	17.0
Interleukin 1- β	5.4	18.5	28.3
Interleukin 6	6.1	24.8	27.9
TNF- α	6.2	23.3	31.2

in the repeated experiments after 6 weeks (17 to 31.2%). Regarding the different cytokines, the highest variation in repeated experiments was observed for TNF- α (31.2%).

Technical Replicate

[Figure 1](#) shows the AUC correlation plots of the two technical replicates (obtained at baseline). The Pearson correlation coefficient R was close to 1 (0.98 or higher) for each cytokine, except interferon- α , which showed a correlation coefficient R of 0.8. [Supplementary Figure 2](#) shows the corresponding Bland-Altman plots for technical replicates.

Biological Replicates

Biological replicates were obtained in only 7 of the 12 participants. [Figure 2](#) shows the AUC correlation plots of the two biological replicates (obtained at baseline). The Pearson correlation coefficients R ranged from 0.75 (interleukin 6) to 0.99 (interleukin 10). [Supplementary Figure 3](#) shows the corresponding Bland-Altman plots for biological replicates.

Stability Over Time

[Supplementary Figures 4–10](#) show the individual concentration-time curves of the seven cytokines (interferon- α , interferon- γ , interleukin 1- β , interleukin 6, interleukin 10, interleukin 8, and TNF- α) at baseline and week 6. [Supplementary Figure 11](#) compares the cytokine responses, expressed as AUCs (pg/mL*h), between the male and female participants. No statistically significant difference was found between male and female participants for any of the seven cytokines at baseline ([Supplementary Figure 11](#)).

[Figure 3](#) shows the AUC correlation plots between the baseline and follow-up experiments at Week 6. The Pearson correlation coefficient R was the lowest for TNF- α (R = 0.63). The highest correlation coefficient was observed for interleukin 10 (0.97). [Supplementary Figure 12](#) shows the corresponding Bland-Altman plots for the repeated experiments. Normalization of cytokine AUCs based on leukocyte counts yielded similar results but did not reduce variability ([Supplementary Figure 13](#)).

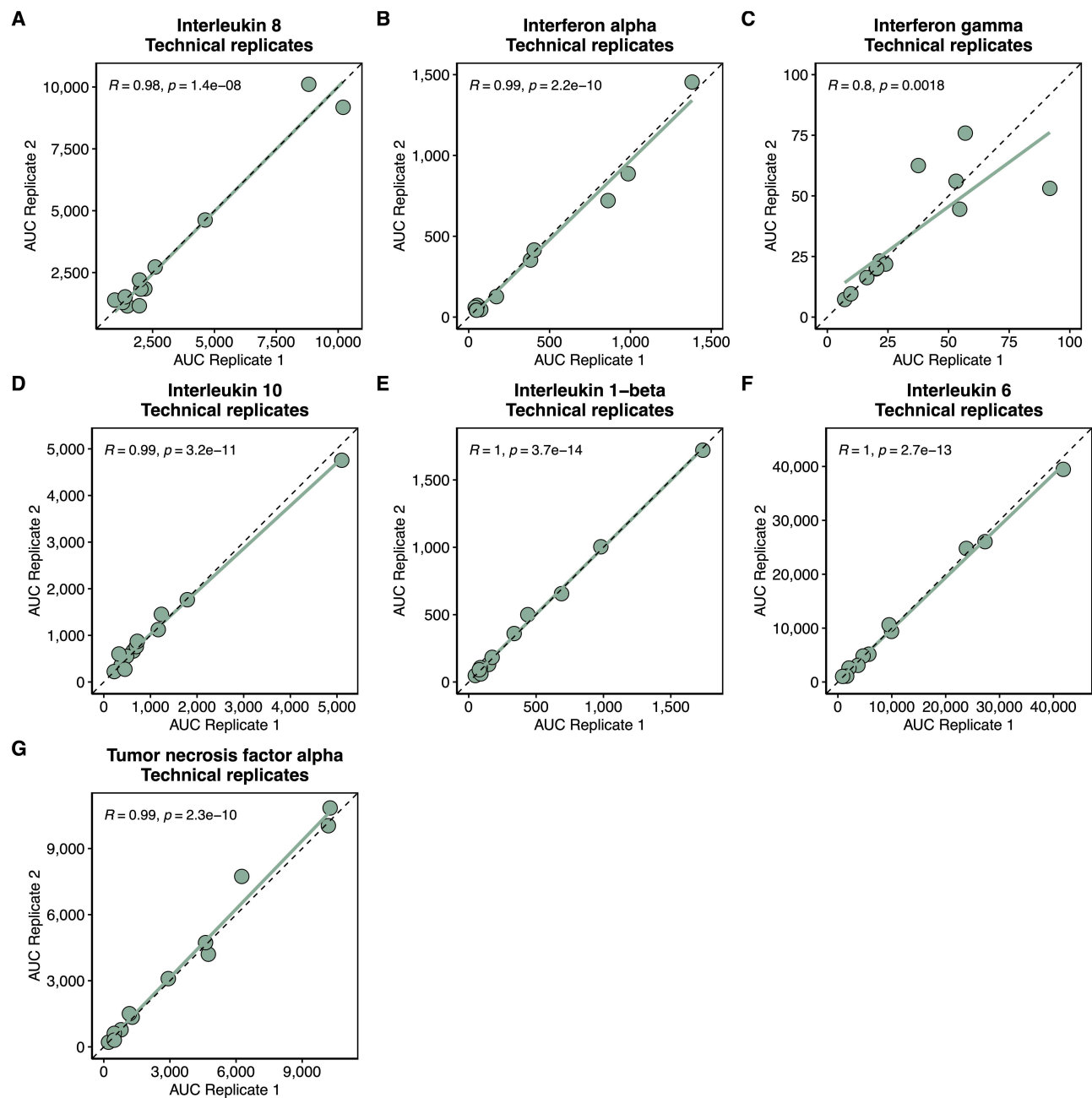


Figure 1 Dot plots assessing the correlation between the individual cytokine AUCs (pg/mL*h) of the technical replicates 1 and 2 at baseline of the ex vivo LPS stimulation. (A) interleukin 8, (B) interferon- α , (C) interferon- γ , (D) interleukin 10, (E) interleukin 1- β , (F) interleukin 6, and (G) TNF- α . The green line is the regression line based on the observed AUCs. Each dot represents one participant. The dashed line indicates a perfect correlation. R is the Pearson correlation coefficient, accompanied by unadjusted p values.

Seasonality

We plotted the AUC values over the seasons ([Supplementary Figure 14](#)). Except for interferon- γ , which peaked in the summer months, the highest AUC values of the remaining cytokines were observed in fall. These associations were not statistically tested.

Cytokine Response and Covid-19 and SARS-CoV-2 Vaccinations

We found no statistically significant difference in the ex vivo cytokine responses at baseline between participants with ($n = 8$) and without ($n = 4$) a history of symptomatic Covid-19 ([Supplementary Figure 15](#)). [Supplementary Figure 16](#) shows the

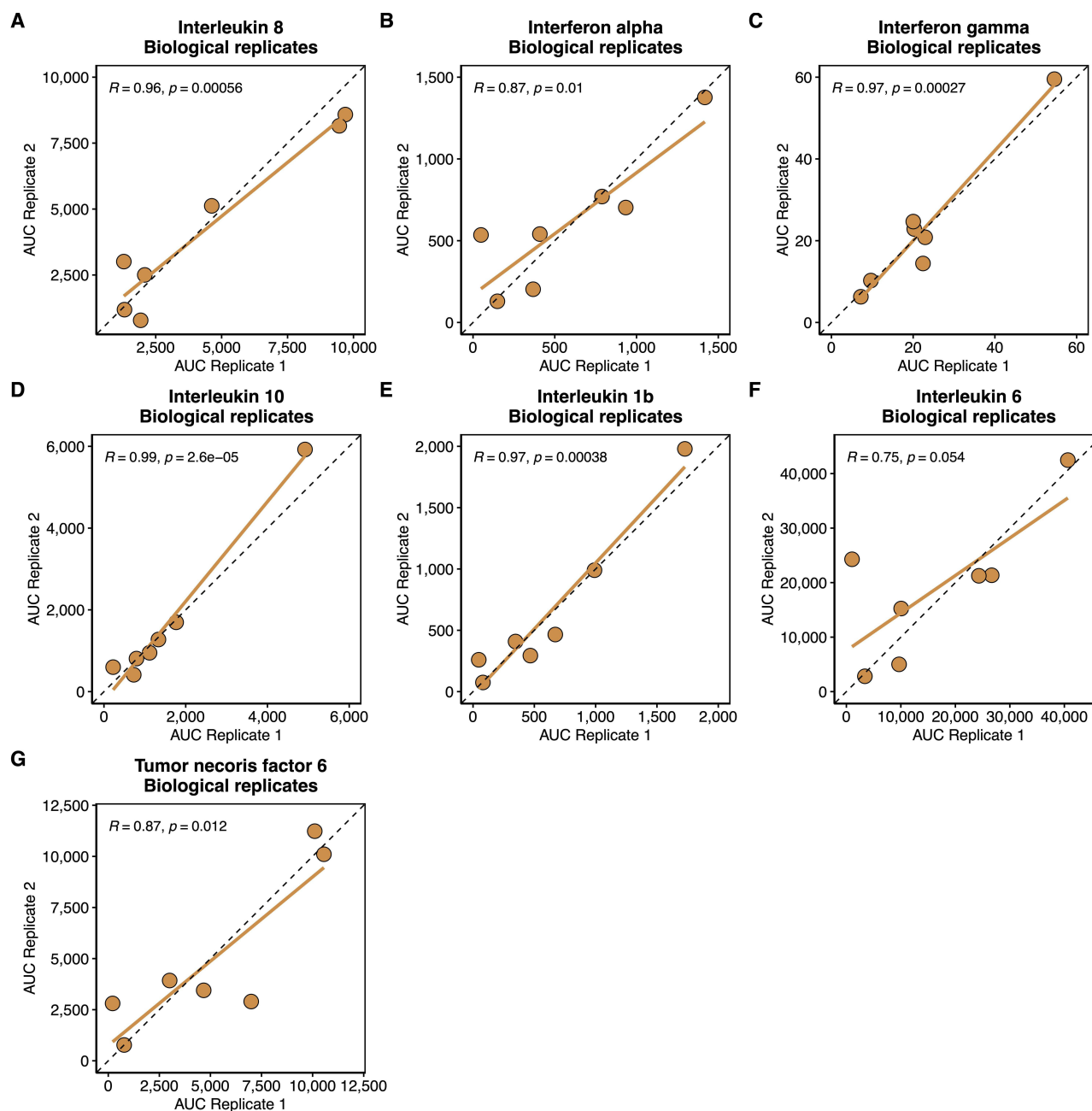


Figure 2 Dot plots assessing the correlation between the individual cytokine AUCs (pg/mL*h) of the biological replicates 1 and 2 at baseline of the ex vivo LPS stimulation. (A) interleukin 8, (B) interferon- α , (C) interferon- γ , (D) interleukin 10, (E) interleukin 1- β , (F) interleukin 6, and (G) TNF- α . The Orange line is the regression line based on the observed AUCs. Each dot represents one participant. The dashed line indicates a perfect correlation. R is the Pearson correlation coefficient, accompanied by unadjusted p values.

correlation between the ex vivo cytokine responses and days since SARS-CoV-2 infection in the 8 participants who reported an infection. All seven cytokines showed a positive correlation between the days since infection and the magnitude of the cytokine response (ie, the more recent the infection, the lower the response). Except for interleukin 8 ($R = 0.76, p = 0.037$), these correlations did not reach statistical significance ([Supplementary Figure 16](#)). None of the cytokines showed a statistically significant correlation with the time since the last SARS-CoV-2 vaccination ([Supplementary Figure 17](#)). The time since the last immunological event (ie, SARS-CoV-2 infection or vaccination, whichever occurred later) showed a statistically significant correlation with interleukin 8 ($R = 0.6, p = 0.043$), Interferon- α ($R = 0.8, p = 0.0032$), interleukin 10 ($R = 0.71, p = 0.012$), and interleukin 6 ($R = 0.62, p = 0.035$) ([Supplementary Figure 18](#)).

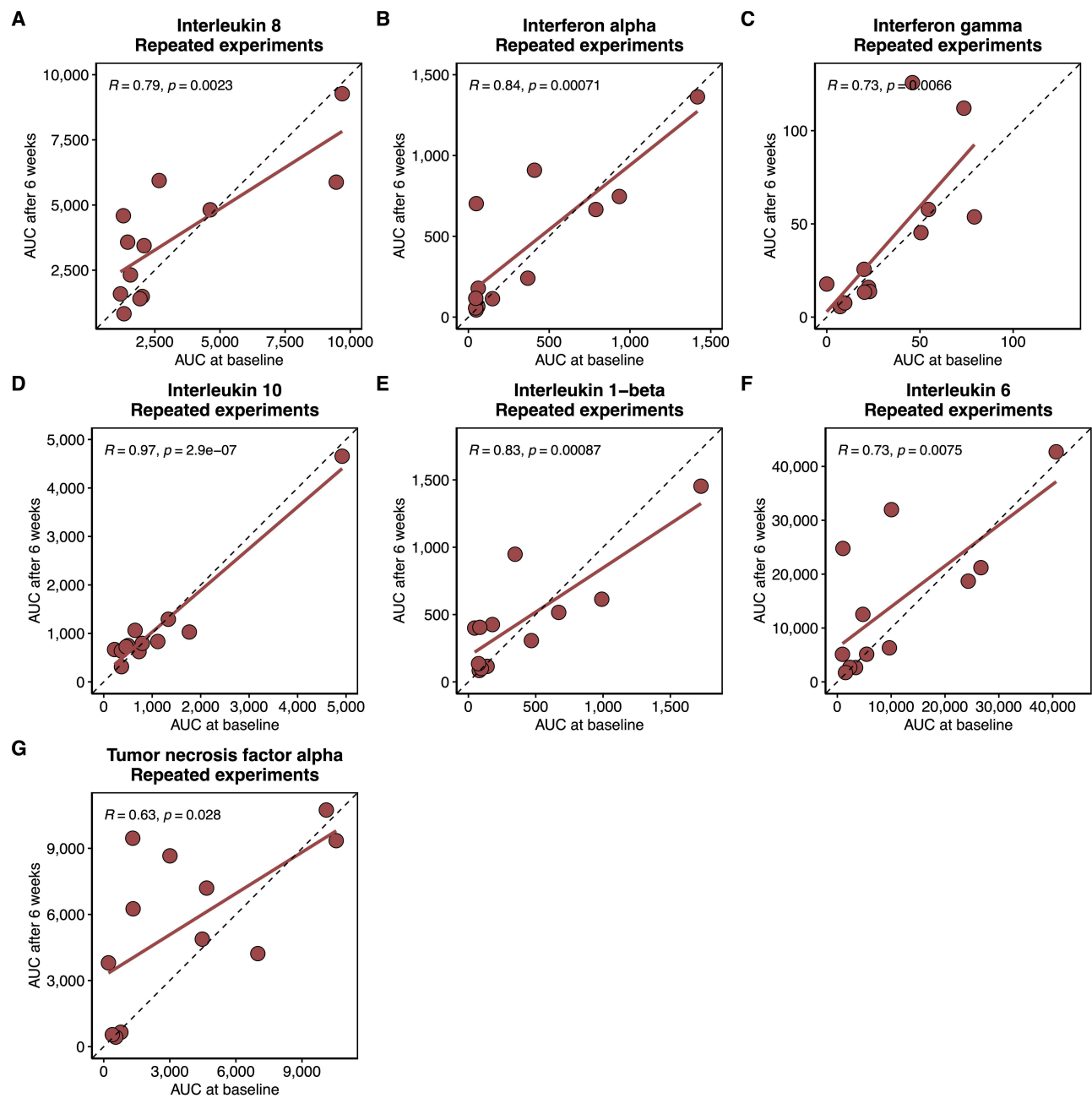


Figure 3 Dot plots assessing the correlation between the individual cytokine AUCs (pg/mL^h) of the baseline and week 6 ex vivo LPS stimulation. **(A)** interleukin 8, **(B)** interferon- α , **(C)** interferon- γ , **(D)** interleukin 10, **(E)** interleukin 1- β , **(F)** interleukin 6, and **(G)** TNF- α . The red line is the regression line based on the observed AUCs. Each dot represents one participant. The dashed line indicates a perfect correlation. R is the Pearson correlation coefficient, accompanied by unadjusted p values.

Discussion

Summary of Findings

Our study evaluated the long-term stability and reproducibility of cytokine responses to ex vivo whole-blood LPS stimulation. By assessing cytokine responses in healthy volunteers at baseline and after a 6-week interval, we observed varying degrees of stability across different cytokines. The technical replicates showed the lowest level of variation, supporting the technical reliability of the Luminex Multiplex assay. As expected, the variation was lowest in the technical replicates (mean coefficients of variation ranging from 5 to 9%), slightly higher in the biological replicates (mean coefficients of variation ranging from 8 to 25%) and highest in the experiments that have been repeated after 6 weeks

(mean coefficients of variation ranging from 17 to 31%). This accumulation of variation is an unavoidable consequence of this experimental design, which should also be taken into account in similar experimental setups. Moreover, biological reproducibility was independent of technical reproducibility. For example, TNF- α had one of the lowest variabilities in technical replicates but one of the highest variabilities in biological replicates.

Regarding the individual cytokines, interleukin 10 and interleukin 8 exhibited the lowest level of variability over time, which indicates that they might be good cytokine candidates for repeated interindividual assessment. In contrast, TNF- α showed the highest variability in the 6-week repeat experiments, making it - despite its immunological relevance - less useful as a biomarker for repeat experiments where minimal variability is desired. On the other hand, TNF- α may be more influenced by time-varying factors, making it a potentially more sensitive biomarker.

Stability Over Time

Our findings build on and extend the existing body of literature in several ways. Van der Linden et al used a whole-blood stimulation system, in which samples were diluted 1:1 with RPMI 1640.⁴ They found intra-individual variations of 15% for TNF- α and 19% for interleukin 10 in experiments repeated after several days. Moreover, van der Linden et al found no interchanging of ranks between high and low producers after repeating the whole blood stimulation on distinct days.⁴ This aligns with our observations on the stability over time of TNF- α and interleukin 10.

Wouters et al, who used RPMI-diluted whole blood and incubated the samples for 18 hours, performed repeated ex vivo LPS stimulations after 1–4 weeks.⁵ They found a low variability for interleukin 1- β and interleukin 6, but a considerable variability for interleukin 8 and TNF- α .

Siedenburg et al examined an even longer interval and found only weak correlations of interleukin 10 and interleukin 1- β responses measured with a five-year time interval ($r = 0.22$ and 0.27 , respectively) and no correlation for TNF- α ($r = 0.06$). It was therefore argued that cytokine reactivity is largely influenced by short-term, intra-individual, or environmental factors rather than by genetic or other time-invariant factors.⁶ Our study partially corroborates this notion, particularly given the observation that variation was lower in the biological (ie, same-day) replicates than in the 6-week repeated experiments.

Seasonality

Ter Horst et al identified seasonality as an important environmental factor that influences the immune response, in addition to specific genetic and nongenetic host factors, such as age and body mass index.^{15,16} However, they stimulated PBMCs instead of whole blood, which makes their findings less comparable to ours than the previously mentioned studies. The circulating biomarkers studied by Ter Horst et al (ie, TNF- α , interleukin 1- β , interleukin 18, interleukin 18 binding protein, and α -1-antitrypsin) were highest in the fall and winter months.¹⁵ This observation was also suggested by our exploratory analysis. Only interferon- γ peaked earlier in the summer months. In contrast, interferon- γ showed only a weak or no seasonal variation in the study of Ter Horst et al. Importantly, we had too few observations, especially in the winter months, to investigate seasonality of inflammatory cytokines reliably.

Sex Differences

The work by Wegner et al found that sex differences in the pro-inflammatory cytokine response to endotoxin unfold in vivo but not ex vivo in healthy volunteers.¹⁷ Accordingly, our data does not identify a significant sex difference, but the sample size was too small to provide sufficient statistical power for this comparison.

Covid-19 and Vaccination

It has been shown that patients with severe Covid-19 exhibit a reduced cytokine response in a whole-blood LPS stimulation assay compared with controls.¹⁸ Another study showed that Covid-19 patients have a diminished ability to produce cytokines in response to ex vivo stimulations with phytohemagglutinin, also indicating an altered or impaired cytokine response in Covid-19.¹⁹ Whether Covid-19 has enduring effects on cytokine responsiveness after acute infection is unknown. We found that subjects with more recent infections had a slight trend towards lower cytokine responses to LPS ex vivo. Moreover, a recent study found that fully vaccinated participants had lower concentrations of inflammatory

markers than unvaccinated participants during and 90 days after symptomatic Covid-19.²⁰ In contrast, Murphy et al found that, upon ex vivo stimulation with unrelated antigens, monocytes of vaccinated individuals produced increased levels of interleukin 1- β , interleukin 6, interleukin 10 but decreased levels of TNF- α , compared with pre-vaccine controls.²¹ By combining vaccination and SARS-CoV-2 infections, we found significant correlations between time since the last immunological event and levels of interleukin 8, Interferon- α , interleukin 10, and interleukin 6. Importantly, these exploratory analyses were not adjusted for multiple comparisons and may represent false positive findings.

Limitations

Our study has several limitations. First, the sample size was relatively small, and all participants were healthy volunteers aged 20 to 30 years, which may limit the generalizability of our findings. Second, we did not control for all potential confounding variables, such as long-term dietary habits, physical activity, or history of Covid-19, which could influence cytokine levels. Third, our study focused on a 6-week interval for repeated measurements, which may be insufficient to capture long-term variability. Fourth, biological replicates were only obtained for 7 of the 12 participants, which could introduce bias in our estimates of biological variability. Fifth, we did not adjust p values for multiple comparisons due to the exploratory nature of this study. It should be emphasized that statistically significant results may be at risk of alpha error and should be interpreted with caution. Finally, there are several other factors, such as shifts in leukocyte subpopulations or epigenetic profiles, that likely influence the ex vivo cytokine response but were not investigated in this study. We believe that this study provides a solid basis for further in-depth analyses of the cellular and (epi-)genetic factors that determine the innate immune response using the ex vivo LPS model.

Conclusions

In conclusion, our study provides insights into the reproducibility and temporal stability of cytokine responses to ex vivo whole blood LPS stimulation. While technical replicates showed high reliability, long-term stability of cytokine responses appears to be influenced by several factors, including time and individual variability. In general, the correlation between baseline and week 6 cytokine responses was high. Because of their low variability, focusing on interleukin 10 or interleukin 8 may be advantageous for repeated ex vivo stimulation assays. In contrast, the high variability of TNF- α makes it a less appropriate candidate for repeated assessment, if high levels of reproducibility are desired. On the other hand, cytokines with high variability may be more sensitive to influencing factors, which may be preferable in certain experiments. Understanding the nuances of cytokine response stability and influencing factors is critical in the design and interpretation of future ex vivo LPS stimulation experiments.

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Disclosure

The authors report no conflicts of interest in this work.

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