University of Groningen

Stability of individual LPS-induced ex vivo cytokine release in a whole blood assay over a five-year interval

Spierenburg, E. A. J.; Portengen, L.; Smit, L. A. M.; Krop, E. J. M.; Hylkema, M. N.; Rijkers, G. T.; Heederik, D.; Wouters, I. M.

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

[Link to publication in University of Groningen/UMCG research database](#)

Technical note

Stability of individual LPS-induced *ex vivo* cytokine release in a whole blood assay over a five-year interval

E.A.J. Spierenburg[a], L. Portengen[a], L.A.M. Smit[a], E.J.M. Krop[a], M.N. Hylkema[b,c], G.T. Rijkers[d], D. Heederik[a], I.M. Wouters[a,*]

[a] *Institute for Risk Assessment Sciences, Division Environmental Epidemiology and Veterinary Public Health, Utrecht University, Netherlands*
[b] *Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands*
[c] *GRIAC- Groningen Research Institute for Asthma and COPD, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands*
[d] *Department of Sciences, University College Roosevelt, Middelburg, the Netherlands*

A B S T R A C T

Objective: In epidemiological and clinical studies, whole blood assay (WBA) has been used as a measure to characterize inter-individual differences in the cytokine response of individuals exposed to inflammatory agents, such as endotoxins. Several short-time repeatability studies have shown stable cytokine levels in individuals over periods of days, weeks or months, but little is known about the long-term stability of cytokine reactivity.
Methods: We studied cytokine response levels in LPS-stimulated whole blood in a cohort of 193 farmers and agricultural industry workers at two time points with a five-year interval.
Results: IL-10 and IL-1β responses measured with a five-year time interval showed a weak positive correlation ($r = 0.22$ and 0.27, respectively), whereas no correlation was observed for TNFα ($r = 0.06$). Cytokine reactivity measured repeatedly at the same time point showed high correlations (IL-10 $r = 0.80$, IL-1β $r = 0.53$ and TNFα $r = 0.74$), suggesting that the observed weak correlations over time are reflective of actual variations in cytokine reactivity over time.
Conclusions: Repeatability of *ex vivo* cytokine reactivity showed to be differential for the measured cytokines, being more stable for IL-10 and IL-1β than for TNFα. However, in general, repeatability of *ex vivo* cytokine reactivity was weak, reflecting that cytokine reactivity can mostly be explained by (short term) intra-individual (immunological) or time varying environmental factors and less by genetic or other time-invariant factors. Therefore, WBA should be regarded as a viable tool to study relationships with current health status and exposure, and only partially as a predictor for a future response.

1. Introduction

The whole-blood stimulation assay (WBA) is a widely used method to study patterns of individual cytokine reactivity in clinical (Segre and Fullerton, 2016) and epidemiological studies (Smit et al., 2009). *In vitro* cytokine response following lipopolysaccharide (LPS) stimulation has been shown to predict clinical outcomes (Segre and Fullerton, 2016), and characterize differences in individual susceptibility (Smit et al., 2009). For instance, an *ex vivo* inflammatory response to LPS in a WBA reflects to a large extent whether individuals are susceptible to adverse respiratory effects induced by high occupational endotoxin exposure (Smit et al., 2009). It has been shown that induced cytokine production is highly reproducible over short periods of time such as weeks or

months (Wouters et al., 2002; Damsgaard et al., 2009) and that it has a genetic component (de Craen et al., 2005). However, it is not clear whether the observed induced cytokine production is consistent over time and represents a stable personal trait or whether cytokine responses merely depend on recent immunological challenges including disease exacerbations, (respiratory) infection episodes and development of allergies.

To investigate the long-term stability of induced cytokine reactivity, we performed a WBA using blood samples collected from the same 193 individuals over an interval of five years. We measured IL-10, IL-1β and TNFα in supernatants of LPS-stimulated whole blood at baseline and after five years of follow-up. Our cohort was set up to study the effect of occupational endotoxin exposure on respiratory health and allergy

(Smit et al., 2009). Cytokine reactivity at baseline was associated with an increased prevalence of endotoxin-related respiratory symptoms. To further evaluate the predictive value of cytokine responsiveness, we need more information on the stability of being a low or high responder in a WBA over an extended period of time.

2. Methods

2.1. Study population

We conducted a five-year follow-up study in endotoxin-exposed farmers and agricultural workers. From the 341 participants at baseline, 193 participated at follow-up. At both time points we collected serum to determine IgE levels and participants filled in a questionnaire on general characteristics, respiratory health, allergies and whether they had experienced a chronic or acute infection, common cold or influenza in the previous week (infection), as described previously (Spierenburg et al., 2017). Atopy was defined as positive specific serum IgE for any of the tested allergens: Grass mix, house dust mite (HDM), cat and dog. Full-shift inhalable dust samples were collected in a subset of participants and analyzed for endotoxin levels by LAL assay. Exposure was then modeled for each participant based on job description (Spierenburg et al., 2017).

The study protocol was approved by the University Medical Centre Utrecht ethics committee and all participants gave written informed consent both at baseline and follow-up.

2.2. Whole blood assay

Both at baseline and at follow-up a WBA with LPS as stimulant was conducted. For the WBA, blood was collected in the morning at the workplace in pyrogen free heparin tubes (BD, Vacutainer) and stored on ice until use. Time between drawing blood and WBA testing was recorded. After transport to the lab, WBA was initiated by transferring 80 μl whole blood to sterile, pyrogen-free U-bottom 96-wells plates (Greiner) and diluted with an equal volume of RPMI with 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen Life Technologies Inc.). LPS (Sigma Aldrich) was added to a final concentration of 1 ng/ml as stimulant. For each participant a sham exposed control was included which contained only RPMI with penicillin and streptomycin. All stimulations were performed in triplicate on the same plate. Plates were incubated for 18 h in a CO_2 incubator(37 °C, 5% CO_2, > 95% relative humidity) after which they were centrifuged (15 min at 1000 g) and supernatants were transferred to 0.5 ml push cap tubes (Micronic Inc.) and stored at −20 °C until cytokine quantification.

2.3. Cytokine quantification

We determined IL-1β and TNFα as these cytokines are known to be involved in endotoxin-induced inflammatory responses. IL-10 was included as a marker of innate cytokine reactivity (Smit et al., 2009).

To determine induced cytokine production, levels of IL-10, IL-1β and TNFα were measured in the supernatants using multiplex technology. For the samples at baseline, we used the in-house Bio-Plex assay from De Jager, et al.(De Jager et al., 2003), and at follow-up the Bio-Plex Pro Assay from Bio-Rad according to manufacturer's protocol. The lower limits of detection (LOD) were 2.3 pg/ml and 0.54 pg/ml for IL-10, 20.8 pg/ml and 6.19 pg/ml for IL-1β and 2.2 pg/ml and 5.86 pg/ml for TNFα for the in-house and Bio-Plex Pro assay respectively. LPS-induced cytokine production was calculated by subtracting cytokine concentrations of unstimulated samples from those of stimulated samples. Unstimulated cytokine concentrations were mostly below LOD both at baseline and follow-up. In a minority of subjects ($n = 0$ at baseline and $n = 5$ for TNFα and $n = 1$ for IL-10 at follow-up) unstimulated cytokine concentrations were higher than stimulated concentrations. In that case, a value of 2/3 of LOD was assigned.

For a subset of 17 samples collected at follow-up we determined the cytokine production in duplicate stimulated wells to determine the repeatability of the WBA stimulation. To test the comparability of the assays, we re-tested a subset of 30 stored samples from baseline with the Bio-Plex Pro Assay from Bio-Rad.

2.4. Data analysis

Samples < LOD were replaced by ⅔ of the lowest detected value per plate, samples > LOD were replaced with 1.5 times the highest detected value per plate. Cytokine levels were ln-transformed for analysis as they were right-skewed.

Time between blood collection and start of WBA averaged 4.1 ± 1.4 h at baseline and 4.5 ± 1.3 h at follow-up. We previously showed an inverse relation between time-to-incubation and cytokine productivity (Smit et al., 2009). At baseline IL-10, IL-1β and TNFα production decreased by 8, 12, and 32% per hour, respectively. At follow-up the decrease was 8, 14 and 29% per hour respectively, based on linear regression analysis for the effect of time-to-incubation with ln-transformed cytokine production adjusted for endotoxin exposure. Cytokine concentrations were adjusted for time-to-incubation prior to further analysis, as was done at baseline (Smit et al., 2009).

We investigated the correlation between baseline and follow-up WBA cytokine production by calculating the Pearson product-moment correlation coefficient. To evaluate whether the variation was due to actual intra-personal variation over time and not due to assay noise, we also calculated correlations for the WBA duplicates at follow-up and re-measured baseline samples.

Additionally we analyzed categorized induced cytokine levels based on tertiles (low, intermediate or high responder) by calculating the kappa (κ, squared weights).

Data was analyzed using SAS 9.4 and R 3.2.2.

3. Results

3.1. General characteristics

General characteristics of the study population at baseline and follow-up remained mostly similar over time (Table 1). However, prevalence of smoking decreased slightly, as did the prevalence of self-reported asthma, while the prevalence of wheeze increased considerably between baseline and follow-up. A more in-depth analysis of the differences in allergy-related variables between baseline and follow-up has been published elsewhere (Spierenburg et al., 2017). We did not find an association between a change in any of the general

Table 1
Characteristics of the study population at baseline and follow-up.

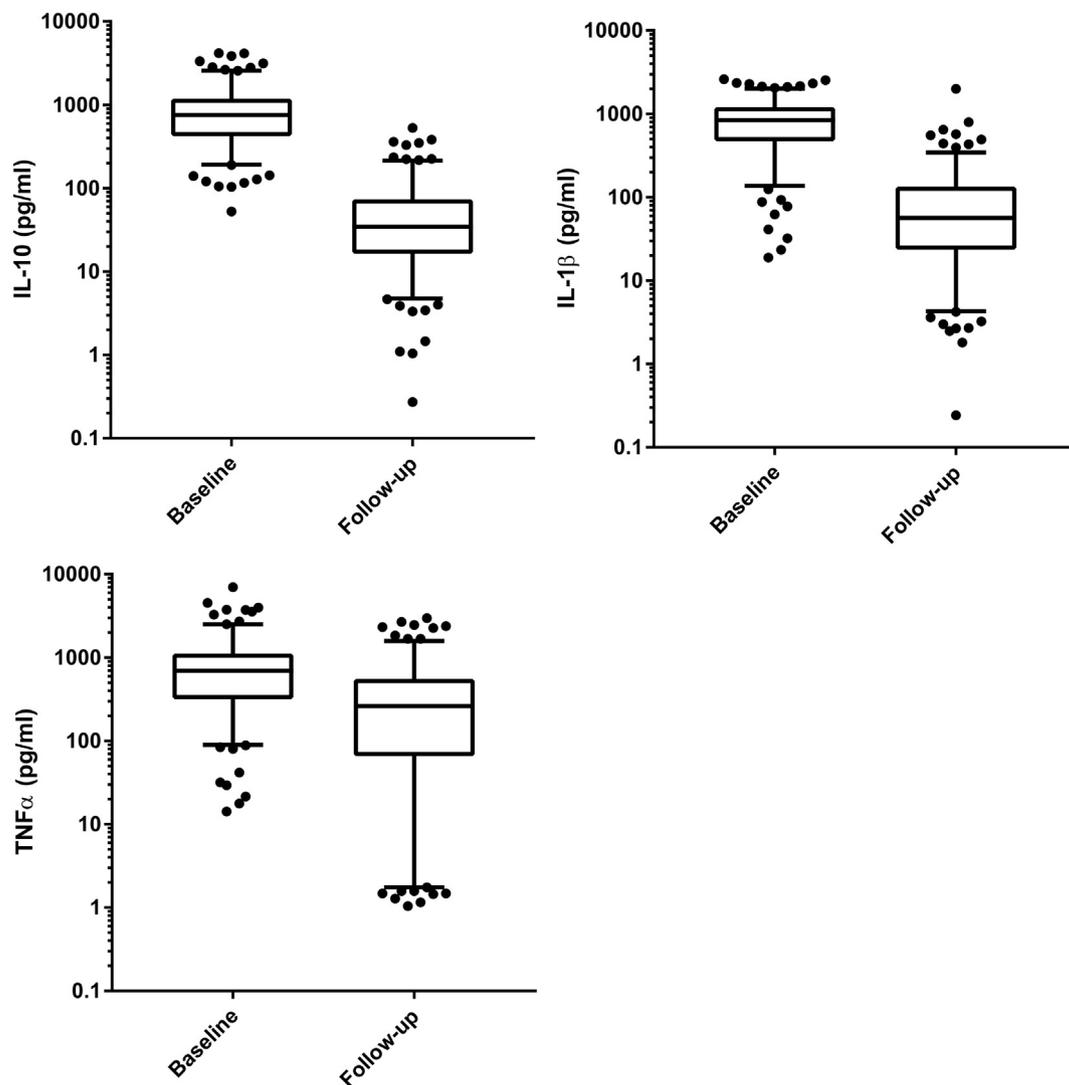|	Baseline	Follow-up
Subjects (n)	193	193
Age* (years; mean (SD))	42.1 (9.2)	46.9 (9.2)
Gender* (female; n (%))	19 (9.8)	19 (9.8)
Farm childhood (n (%))*	109 (56.5)	105 (54.7)
Smoking (n (%))*	49 (25.4)	43 (22.4)
Endotoxin exposure (EU/m³; GM (GSD))	298 (4.6)	266 (5.0)
Asthma (n (%))*	18 (9.3)	10 (5.2)
Wheeze (n (%))*	24 (12.4)	36 (18.8)
Infection (n (%))*	61 (31.6)	58 (30.1)
Allergy (n (%))*	57 (29.5)	58 (30.2)
Hay fever (n (%))*	27 (14.0)	28 (14.6)
Total IgE(IU/ml; GM (GSD))	24.8 (7.2)	23.2 (6.3)
Atopy (n (%))	63 (32.6)	65 (33.7)
Grass IgEpositive (n (%))	40 (20.7)	41 (21.2)
HDM IgEpositive (n (%))	44 (22.8)	41 (21.2)
Cat IgEpositive (n (%))	4 (2.1)	6 (3.1)
Dog IgEpositive (n (%))	2 (1.0)	3 (1.6)

* Self-reported

Fig. 1. Induced cytokine concentrations at baseline and follow-up. Absolute cytokine levels are shown as 5-95th percentile box and whisker plots.

characteristics and change in z-score of cytokine expression (data not shown).

3.2. Levels of cytokine response at baseline and follow-up

To compare absolute cytokine response levels between baseline and follow-up and between the two analysis methods, we determined induced cytokine response levels at both time points. A significant difference was observed between induced cytokine production at the two time points (p < .0001 for all cytokines; Fig. 1). In unstimulated samples, cytokines were usually below limit of detection at both time points. In those cases where cytokine concentrations were measurable in unstimulated samples, a similar absolute level difference between the two time points is observed. It is likely that the absolute difference in cytokine levels at baseline and follow-up is partly due to a difference in assays used, as the cytokine concentrations of baseline samples re-measured at follow-up with the Bio-Plex Pro assay were in the same range as the cytokine levels of the follow-up samples measured with the same assay. Moreover, moderate to good correlation was found between cytokine levels determined at baseline and re-measured at follow-up, see Fig. 2. This indicates that although we used two different assays, the influence on the correlation between the two time points is limited.

3.3. Correlation between cytokine response at baseline and follow-up

To estimate the stability of cytokine reactivity over time we tested correlations between the cytokine levels in the WBA of the 193 participants included both at baseline and follow up. We observed weak but positive correlations between cytokine levels at baseline and follow-up for IL-1β ($r = 0.27$, 95%CI: 0.13–0.39) and IL-10 ($r = 0.22$, 95%CI:0.08–0.35), whereas there was no correlation for TNFα ($r = 0.06$, 95%CI: -0.08-0.20; Fig. 3). Evaluation of categorized induced cytokine levels based on tertiles showed similar results for all three cytokines: IL-1β $\kappa = 0.206$, IL-10 $\kappa = 0.134$ and TNFα $\kappa = 0.052$ (see Table 2 for contingency table). The majority of participants either remained in the same response class (39%, 40% and 46% of participants for IL-10, IL-1β and TNFα respectively) or changed to the adjacent class from baseline to follow-up (44%, 44% and 46% for IL-10, IL 1β and TNFα respectively). A change from lowest to highest tertile or *vice versa* was less common and occurred in 17%, 16% and 18% of participants for IL-10, IL 1β and TNFα respectively.

To get an insight into the assay reproducibility, we looked into the correlation between duplicate samples. These correlations were moderate to high: IL10 $r = 0.80, p < .0001$; IL-1β $r = 0.53, p < .03$; TNFα $r = 0.74, p < .001$, indicating that the observed correlations between $t = 0$ and $t = 5y$ will for a large part be due to actual differences over time.
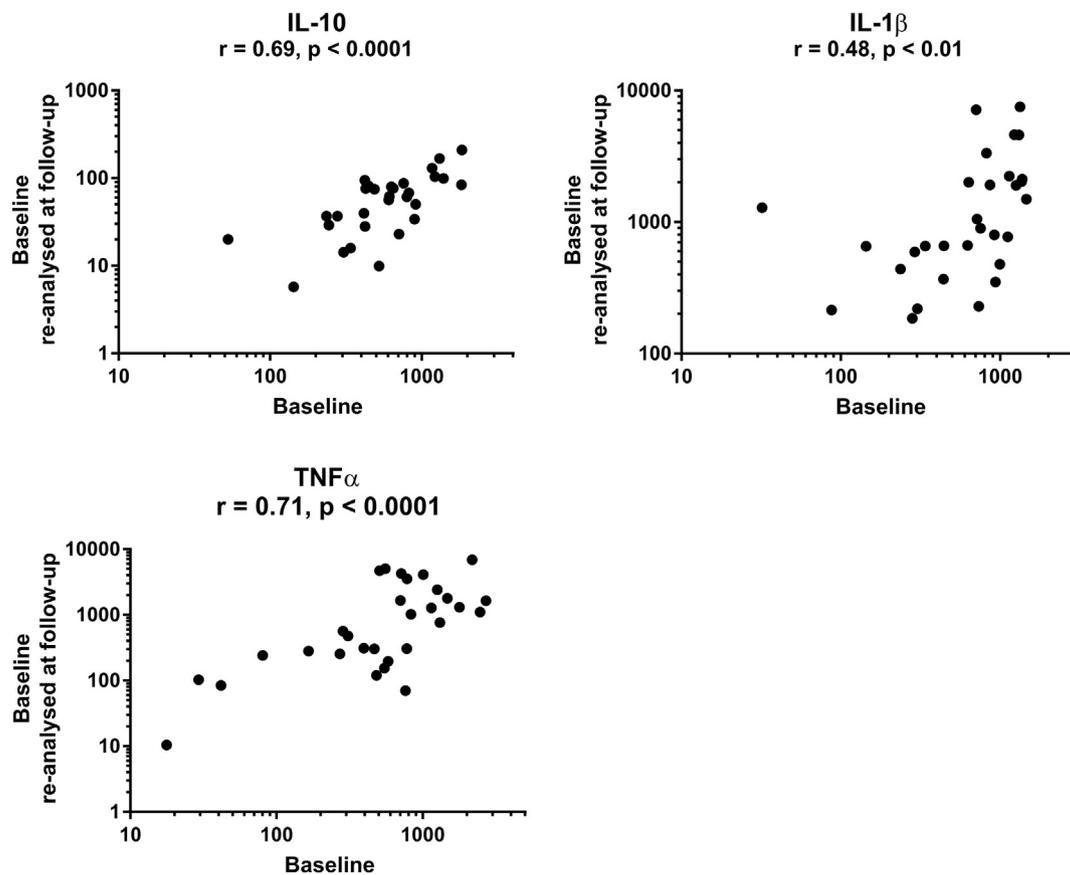
Fig. 2. Cytokine levels of 30 samples from baseline, analyzed at baseline and re-analyzed with the new assay at of follow-up. Scatterplot with correlation coefficient (r) and *p*-value. Cytokine levels presented in pg/ml.

4. Discussion

In this study, we determined the stability of cytokine expression over time by correlating measured induced cytokine responses in 193 individuals at two time points with a five year interval. In general, stability over time was limited, yet LPS responsiveness for IL-10 and IL-1β showed to be somewhat more stable over time than TNFα. Research by May, et al.(May et al., 2009) observed a similar result in a study with follow-up time of 1 year for IL-10 and TNFα (IL-10: $r = 0.48$, p < .001; TNFα: $r = 0.15$, $p = .087$), suggesting that TNFα may be more variable in general, while induced IL-10 production is more stable. This implies that IL-10 reactivity better reflects an innate inflammatory tendency while TNFα may predominantly reflect influence of recent immunological challenges.

There are several factors which may impact short term cytokine reactivity and thus may account for the low degree of correlation between two measurements with a five-year interval. First, there are methodological factors. In previous studies, several methodological factors have been reported to influence observed cytokine reactivity such as the number of cells in the assay (Hartmann et al., 2016), time-to-incubation (Bakiyeva et al., 2005), storage conditions of blood samples before usage in WBA and even the type of plastic ware used (Hartmann et al., 2016). We were aware of these prior to this study, therefore, to minimize the effect of methodological differences, we used the same protocol and type of plastic ware at both time points. However, as a fixed volume of blood was used per stimulation, the number of cells for each WBA was not controlled for as the cell number was not available at follow-up. A possible intra-individual difference in cell count may have introduced some variation. However, we performed a cell count for the samples at baseline and adjustment for the cell number in the baseline analyses only slightly attenuated the

associations between endotoxin exposure and IL-1β and IL-10 (Smit et al., 2009).

Another methodological factor to be considered is the influence of varying time-to-incubation. Several studies have shown that cytokine responsiveness is inversely related to time-to-incubation (Smit et al., 2009; Van Der Linden et al., 1998; Egger et al., 1997). We took this into account by applying modeled correction factors to the cytokine response prior to the analyses. Basis of this correction factor is the assumption that time to incubation is log-linearly associated with cytokine levels, while in fact the actual decline in cytokine responsiveness might follow a different course such as an exponential curve. However, a sensitivity analysis calculating the correlations with the unadjusted cytokine responses did only marginally change the results, which indicates that even though there is an effect of time-to-incubation on whole blood cytokine reactivity, the overall correlation between cytokine responses is a robust one.

Non-methodological factors which may impact repeated cytokine reactivity are time varying environmental and intra-individual (immunological) factors. Endotoxin exposure has previously been shown to influence cytokine reactivity (Castellan et al., 1984; Smit et al., 2011), but also glucans (Wouters et al., 2002) and other exposures may induce a cytokine response *in vivo*, both in the short and long term. As endotoxin exposure has been shown to influence *ex vivo* cytokine reactivity, it might be of influence on the correlations found between the time points in this study. However, there was no association between change in endotoxin exposure and change in cytokine reactivity. Immunological factors that might influence cytokine reactivity are (recent) infections and immune modulating medication. We did not have information on medication use, yet we did have self-reported information on infections in the week prior to WBA. Reported chronic or acute infection, common cold or influenza in the week prior to WBA
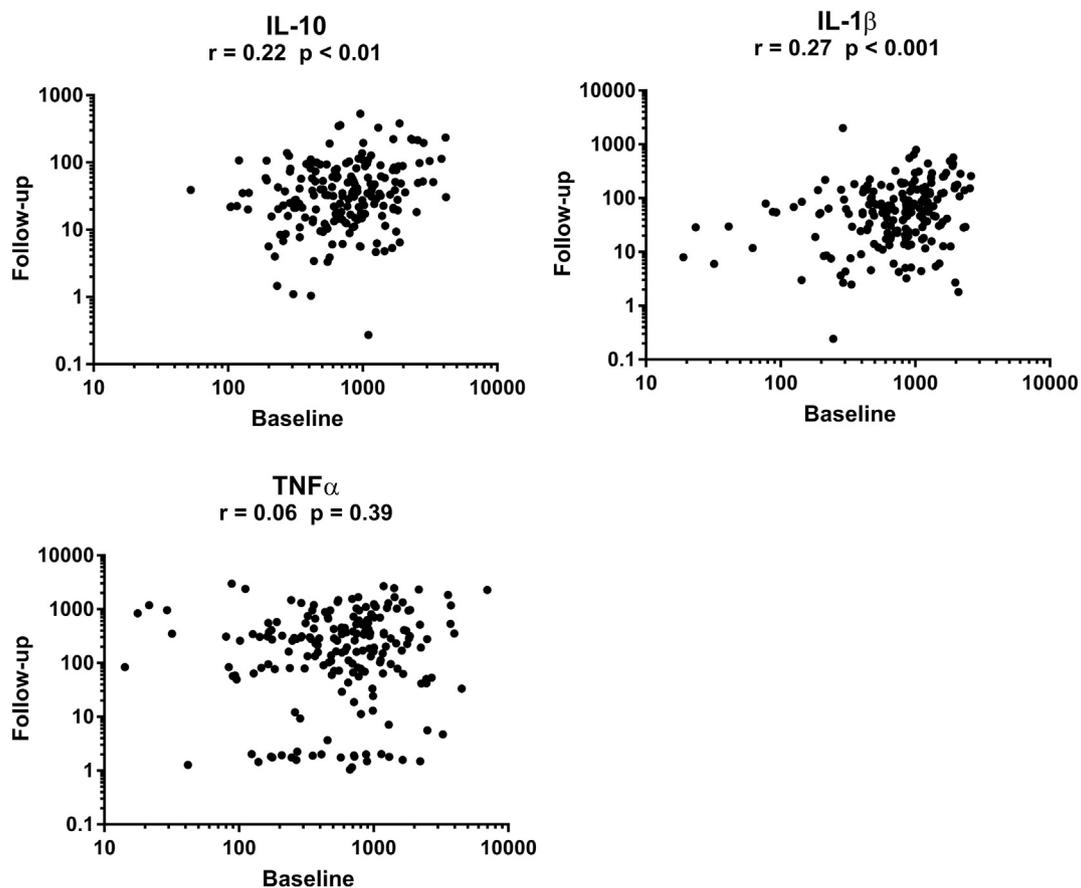
Fig. 3. Scatterplot with correlation coefficient (r) and *p*-value for cytokine levels of 193 participants at baseline and follow-up. Cytokine levels presented in pg/ml.

was however not associated with cytokine responses, and thus is not a likely explanation for the observed weak correlations over time. Additionally a change in atopy status was not associated with a change in cytokine reactivity. Another intra-individual factor which might influence cytokine reactivity is smoking. However, at baseline we did not find an association between smoking habit and any of the cytokines (Smit et al., 2009). Also in the current study we did not find a correlation between change in smoking habit and change in cytokine responsiveness.

A shortcoming of our study is the substantial drop out of participants between baseline and follow-up: 43% of the participants were lost to follow-up (included in follow-up (FU) $n = 193$; lost to follow-up (LTF) $n = 148$). This may have led to selection bias, although this is of little importance for the present research question on intra-individual repeatability of cytokine responsiveness. We previously reported that there is no evidence of healthy worker selection based on atopic sensitization, lung function and respiratory symptoms (Spierenburg et al., 2015). In this study, we did not observe any evidence of a healthy worker selection based on induced cytokine production. Baseline cytokine levels were similar for those LTF and those included in FU (IL-

10: LTF 439 ± 2.5 pg/ml, FU 479 ± 2.3 pg/ml, $p = .35$; IL-1β: LTF 420 ± 2.2 pg/ml, FU 419 ± 2.3 pg/ml, $p = .97$ and TNFα: LTF 148 ± 4.1 pg/ml, FU 167 ± 3.1 pg/ml, $p = .36$).

Organic dust exposure has been linked to adverse respiratory effects and a protective effect on allergy (Smit et al., 2008), and endotoxin sensitivity is known to vary from person to person (Kline et al., 1999). With the immune system being an important link between organic dust exposure and its possible health effects, it is a logical factor to contribute to these interpersonal differences. Most studies focusing on immunological inter-personal differences as assessed by WBA present it as a personal characteristic and thereby implicitly assume this is a more or less stable trait over longer periods of time. The genetic make-up could underlie this personal characteristic and several studies investigated a possible link between cytokine responses and candidate genes, showing that there is indeed a relationship between certain genetic markers and induced cytokine production (Westendorp et al., 1997) and between genetic markers and endotoxin related health outcomes (Smit et al., 2011). We did indeed find weak positive correlations between cytokine levels over a period of 5 years for IL-1β and IL-10. This implies that cytokine reactivity has at least some stability over

Table 2

Contingency table showing number of participants categorized by induced cytokine reactivity at baseline and follow-up, based on tertiles per time-point.

		Follow-up								
		IL-10			IL-1β			TNFα		
		Low	Med	High	Low	Med	High	Low	Med	High
Baseline	Low	26	19	19	26	25	14	25	23	16
	Med	24	22	18	23	21	20	21	20	24
	High	14	24	27	16	17	31	19	21	24

time, which suggest a partial role of genetic factors. The marginal stability of cytokine levels found in the current study are in line with a study of 210 twins where the normalized heritable influence in serum proteins was estimated to be < 0.25 for IL-10 and < 0.40 for IL-1β and TNFα (Brodin et al., 2015). Although the repeatability over time is underestimated because of methodological variation, we found reasonably good assay reproducibility that could not fully explain the limited correlation over five years. Therefore, we conclude that there is substantial intra-individual variation in responsiveness over time, which is likely due to triggers with more acute effects. Although in our study we did not find a relation between recent, self-reported infections in the previous week and cytokine reactivity, there may be other factors such as environmental factors or more severe immunological challenges that impact cytokine reactivity in the short term. A study by Castellan, et al. (Castellan et al., 1984) already showed an attenuation of the cytokine reactivity in response to repeated endotoxin exposure, showing that although there is a relation between environmental factors and cytokine reactivity, this is less a stable state, and more an adaptive feedback-loop.

In conclusion, cytokine responsiveness of IL-1β and IL-10 are to some extent stable over a 5-year time period, while TNFα is more variable. This suggest that investigating cytokine responsiveness in relation with health outcomes is only partly predictive for future responses, yet may provide insight in immune regulation underlying current observed responses.

Acknowledgements

Funding

Conflict of interest

All authors have no conflict of interest to declare.

References

Bakiyeva, L.T., Brooks, R.A., Rushton, N., 2005. Inter-individual and intra-individual variability in TNF-alpha production by human peripheral blood cells in vitro. Cytokine 30 (1), 35–40.

Brodin, P., Jojic, V., Gao, T., Bhattacharya, S., Angel, C.J.L., Furman, D., et al., 2015. Variation in the human immune system is largely driven by non-heritable influences. Cell 160 (1–2), 37–47.

Castellan, R.M., Olenchock, S.A., Hankinson, J.L., Millner, P.D., Cocke, J.B., Bragg, C.K., et al., 1984. Acute bronchoconstriction induced by cotton dust: dose-related responses to endotoxin and other dust factors. Ann. Intern. Med. 101 (2), 157–163.

Damsgaard, C.T., Lauritzen, L., Calder, P.C., Kjær, T.M.R., Frøkiær, H., 2009. Whole-blood culture is a valid low-cost method to measure monocytic cytokines - a comparison of cytokine production in cultures of human whole-blood, mononuclear cells and monocytes. J. Immunol. Methods 340 (2), 95–101.

de Craen, A.J.M., Posthuma, D., Remarque, E.J., van den Biggelaar, A.H.J., Westendorp, R.G.J., Boomsma, D.I., 2005. Heritability estimates of innate immunity: an extended twin study. Genes Immun. 6 (2), 167–170.

De Jager, W., Te Velthuis, H., Prakken, B.J., Kuis, W., Rijkers, G.T., 2003. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin. Diagn. Lab. Immunol. 10 (1), 133–139.

Egger, G., Kukovetz, E.M., Hayn, M., Fabjan, J.S., 1997. Changes in the polymorphonuclear leukocyte function of blood samples induced by storage time, temperature and agitation. J. Immunol. Methods 206 (1–2), 61–71.

Hartmann, S.B., Emnéus, J., Wolff, A., Jungersen, G., 2016. Revisiting the IFN-γ release assay: whole blood or PBMC cultures? - and other factors of influence. J. Immunol. Methods 434, 24–31.

Kline, J.N., Cowden, J.D., Hunninghake, G.W., Schutte, B.C., Watt, J.L., Wohlford-Lenane, C.L., et al., 1999. Variable airway responsiveness to inhaled lipopolysaccharide. Am. J. Respir. Crit. Care Med. 160 (1), 297–303.

May, L., van Bodegom, D., Kuningas, M., Meij, J.J., de Craen, A.J.M., Frölich, M., et al., 2009. Performance of the whole-blood stimulation assay for assessing innate immune activation under field conditions. Cytokine 45 (3), 184–189.

Segre, E., Fullerton, J.N., 2016. Stimulated whole blood cytokine release as a biomarker of immunosuppression in the critically ill: the need for a standardized methodology. Shock 45 (5), 490–494.

Smit, L.A.M., Heederik, D., Doekes, G., Blom, C., Van Zweden, I., Wouters, I.M., 2008. Exposure-response analysis of allergy and respiratory symptoms in endotoxin-exposed adults. Eur. Respir. J. 31 (6), 1241–1248.

Smit, L.A.M., Heederik, D., Doekes, G., Krop, E.J.M., Rijkers, G.T., Wouters, I.M., 2009. Ex vivo cytokine release reflects sensitivity to occupational endotoxin exposure. Eur. Respir. J. 34 (4), 795–802.

Smit, L.A.M., Heederik, D., Doekes, G., Koppelman, G.H., Bottema, R.W.B., Postma, D.S., et al., 2011. Endotoxin exposure, CD14 and wheeze among farmers: a gene - environment interaction. Occup. Environ. Med. 68 (11), 826–831.

Spierenburg, E.A.J., Smit, L.A.M., Heederik, D., Robbe, P., Hylkema, M.N., Wouters, I.M., 2015. Healthy worker survivor analysis in an occupational cohort study of Dutch agricultural workers. Int. Arch. Occup. Environ. Health 88 (8), 1165–1173.

Spierenburg, E.A.J., Smit, L.A.M., Krop, E.J.M., Heederik, D., Hylkema, M.N., Wouters, I.M., 2017. Occupational endotoxin exposure in association with atopic sensitization and respiratory health in adults: results of a 5-year follow-up. PLoS One 12 (12).

Van Der Linden, M.W., Huizinga, T.W.J., Stoeken, D., Sturk, A., Westendorp, R.G.J., 1998. Determination of tumour necrosis factor-a and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. J. Immunol. Methods 218 (1–2), 63–71.

Westendorp, R.G.J., Langermans, J.A.M., Huizinga, T.W.J., Verweij, C.L., Sturk, A., 1997. Genetic influence on cytokine production in meningococcal disease [11]. Lancet 349 (9069), 1912–1913.

Wouters, I.M., Douwes, J., Thorne, P.S., Heederik, D., Doekes, G., 2002. Inter- and intraindividual variation of endotoxin- and β(1 → 3)-glucan-induced cytokine responses in a whole blood assay. Toxicol. Ind. Health 18 (1), 15–27.