Cytokine responses to lipopolysaccharide in vivo and ex vivo : Genetic polymorphisms and inter-individual variation

Schippers, E.F.

CHAPTER 4

TNF-α and IL-10 production upon whole blood stimulation with a wide range of LPS concentrations: estimating an individual's dose-response characteristic described by the underlying receptor-ligand model.

E.F. Schippers, C.A.E. Martina, H. Mattie, J.T. van Dissel.

Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands

Submitted

Abstract

Individuals are commonly described as 'low' or 'high' producers of cytokines based on ex vivo stimulation of their cells with various stimuli, most often lipopolysaccharide. This dichotomous characteristic is subsequently used as reference in clinical and genetic studies. Since this approach is not based on a physiological model and studies using this approach yielded conflicting results, we investigated whether it was possible to better describe an individuals cytokine production profile using a receptor-ligand interaction model that is fully characterized by two parameters, i.e., EC_{50}, the estimated LPS concentration at which half of the cytokine concentration is reached and the E_{max}, the estimated maximal concentration of cytokine released. Repeated measurements over several weeks disclosed that these two parameters were highly constant for individuals, yet differed between individuals. In contrast with this, only the TNF-α concentration measured after stimulation with the lowest LPS concentration (0.1 ng/mL) was different between the subjects, whereas IL-10 concentrations were only different between the subjects after stimulation with the lower LPS concentrations (i.e. 0.1 through 100 ng/mL). Our findings suggest that relevant information on cytokine release is lost, when the commonly applied approach is taken, i.e. testing the TNF-α and or IL-10 release after stimulation with a single, and often high, LPS concentration.

Studies aimed at correlating in vitro with in vivo phenotypes and genetic polymorphisms should for this purpose use large dose-response ranges of whole blood stimulation with LPS to be able to closely estimate these dose-response characteristics. Such dose-response characteristics are more likely to be good estimates of an individual's in vitro cytokine production profile and therefore more suitable as a reference used in studies that investigate the genetic factors that make up for the large inter-individual variation found in cytokine production upon whole blood stimulation with LPS.

Introduction

Ex vivo cytokine production capacity of an individual is elegantly determined in the supernatant of whole blood after stimulation with lipopolysaccharide (LPS). The system provides a natural environment for cytokine producing cells, mainly being monocytes. Based on a rather small day-to-day intra-individual variation and laboratory variation, both estimated at around 15%, in combination with a rather large inter-individual variation, the system is able to distinguish 'high' and 'low' producing individuals of TNF-α and IL-10 based on the cytokine concentration found in the supernatant upon stimulation with one single concentration of LPS (1). In one study the aforementioned approach was able to detect differences in ex vivo cytokine production in relatives of patients with poor or fatal outcome of meningococcal disease as compared to relatives of patients with more favorable outcome (2). These observations led to the conclusion that the inter-individual variation found in these cytokine concentrations is to be explained to a large extend by genetic background (2;3). Many studies set out to search for genetic factors explaining this variation. Better understanding of the pathways involved in LPS sensing and intracellular signaling were essential in the search for these genes. Studies mainly focused on finding associations between specific gene(-promoter) polymorphisms and (whole blood) stimulation phenotype (i.e. individuals categorized as 'high' as compared to 'low' producers) based on a cytokine measurement after stimulation with one single – but between studies varying – concentrations of LPS (2-30). The conclusions of these studies have been conflicting. Part of the explanation for this might be differences in the handling of samples, differences in the various assays, cellular sources (whole blood, isolated cells), the LPS composition, incubation times, and, likely most importantly the differences in the LPS concentrations used to stimulate the cells. Categorizing individuals as 'high' as compared to 'low' producers based on cytokine production upon a single LPS concentration might not be adequate. First, the LPS concentrations used in these studies almost always exceeded the concentrations found in patients with infectious disease, hampering the translation of the in vitro phenotype to the in vivo situation. Secondly, the cytokine concentrations found after stimulation with a single high concentration of LPS will reflect the maximal production capacity, but might not necessarily provide insight in the cytokine response at lower LPS concentrations. Furthermore, aspects of the dose-response characteristics, for instance the LPS concentration at which 50% of the maximal cytokine concentration is reached and the maximal cytokine production capacity of the dose-response curve are not taken into account. Since the discovery of the toll-like receptor 4, the main extra cellular receptor for LPS, these aspects are of increased interest since this

mode of LPS signaling is likely to follow the basic concepts of the receptor-ligand model used in other fields of research (31). Based on the dose response model know as the C_{max} model we calculated the dose response characteristics of individuals by using supernatant cytokine concentrations after stimulation with a wide range of LPS concentrations. In order to be able to quantify the influence of the laboratory and the intra-individual day-to-day variation on these parameters we performed each measurement separately in duplicates and repeated the assay several times over several weeks. We hypothesize that the intra-individual day-to-day variation in the dose-response parameters is small as compared to the variation found in the intra-individual day-to-day variation found in a single point measurement and have better discriminative properties in labeling an individuals phenotype. If these premises were met, it would be suitable to use these parameters as patient characteristics in future research.

Materials and Methods

Subjects and blood sample collection.

We studied two healthy male subjects. Neither of the subjects used any prescription drugs, nor where they active smokers. No dietary restrictions were applied on the day of blood collection nor on the days prior. In the 3 weeks before and during the sampling the subjects did not experience any signs or symptoms of infection such as fever. Blood samples were drawn at 9:00 am using a Vacutainer® blood collecting system in four endotoxin-free collection tubes (Endo Tube ET, 4 mL, containing 120 IU sodium heparin, Chromogenix, Amsterdam, Netherlands), through puncture of a large antecubial vene. From each tube a separate whole blood stimulation assay, i.e. two whole blood stimulations for each cytokine, was performed without delay as described below. The whole procedure was repeated 7-9 times over a 3-week period.

Whole blood stimulation assay and LPS preparation.

Briefly, whole-blood samples were mixed 1:1 with RPMI 1640 (Gibco, Germany) and LPS (*Escherichia coli* O111, Sigma) diluted in RPMI 1640 was added to final concentrations of 0.1, 1, 10, 100, 1000 and 10,000 ng/mL and a total volume of 1 ml using 24-well microtiter plates. On each day fresh LPS dilutions were prepared from one single batch of LPS (5 mg/ml, stored at -80 ^{0}C). The stimulations were performed by incubation of the 1:1 diluted blood without LPS addition as a negative control. Cells were subsequently stimulated for 4 and 24 hours at 37 ^{0}C under 5% CO_2 for determination of TNF-α and IL-10, respectively. Supernatants (4 times 200 µl for each LPS concentration) were collected after

centrifugation of the microtiter plates at 600 x *g* at 4 ^{0}C and stored at -70 ^{0}C in 250 µl aliquots for final measurement of the cytokines.

TNF-α and IL-10 measurement.

Cytokine concentrations in the supernatants derived from the repeated stimulation assays were analysed on a single day using one ELISA batch. Tumor necrosis factor (TNF)-α and Interleukin-10 concentrations were determined with a standard ELISA technique (PeliKine Compact^{TM}, Central Laboratory of the Netherlands Red Cross Blood transfusion Service, Amsterdam, The Netherlands); the lower detection limit was 4.0 pg/mL. Each cytokine concentration was measured in duplicate. In case of laboratory failure, supernatants were retested using a new, previously unfrozen aliquot. In such case all supernatants derived from the two whole blood stimulations from that single vena puncture were retested.

Calculation of ex vivo TNF-α, IL-10 and LPS dose-response.

Dose-response characteristics for the mean of the whole blood stimulation for each cytokine (i.e. TNF-α or IL-10) were calculated for each subject using the ligand-receptor response model as previously described (31). The basis for this model is the principal that the interaction between a ligand (i.e., LPS) and its receptor is the first step leading to the response. The effect is correlated with the number of receptors occupied and is maximal when all receptors are occupied. The ligand concentration at which 50% of the maximal response is reached (EC_{50}) is a parameter of the affinity of the ligand for its receptor. Taking together, the assumptions lead to the E_{max} model, mathematically described as the Hill equation.

In the first approach we used a single step E_{max} equation as a model.

$$E_N = E_{N,max} \text{ x } C/(EC_{50} + C) \qquad \text{(model 1)}$$

Where E_N is the observed cytokine concentration at a given LPS concentration C, EC_{50} is the estimated LPS concentration at which 50% of the maximal cytokine concentration is reached, and $E_{N,max}$ is the estimated maximal cytokine concentration.

In a second approach, for instance if we assumed that the release of cytokine might be described better as a two-stage process; first, stimulation leads to the release of preformed cytokines, stored intracellular in granules; secondary, stimulation activates gene transcription and de novo production of cytokines. Furthermore, the measured amount of cytokine released upon stimulation with LPS is most likely a sum of cytokine released by stimulation of different pathways. Together, this would result in a two-step release, with

different time coefficients and possibly different regulatory set points, depending on the intensity of the stimulus. To fit for such a model, the second analysis was based on two Emax equations revealing four dose-response characteristics for the LPS-induced cytokine production (i.e. TNF-α or IL-10) in each subject.

$$E_N = E_{N,max\text{-}1} \text{ x } C/(EC_{50\text{-}1} + C) \quad + \quad E_{N,max\text{-}2} \text{ x } C/(EC_{50\text{-}2} + C) \qquad \text{(model 2)}$$

Where E_N is the observed cytokine concentration at a given LPS concentration C, $EC_{50\text{-}1}$ and $EC_{50\text{-}2}$ are the estimated LPS concentration at which 50% of the maximal cytokine concentration at the first stage and the second stage was reached, respectively, and $E_{N,max\text{-}1}$ and $E_{N,max\text{-}2}$ is the estimated maximal cytokine concentration at the first stage and the second stage, respectively.

The characteristics were calculated by non-linear regression using SPSS 12.0.1.

Statistical Analysis.

The spread in the TNF-α and IL-10 ELISA measurements was estimated by calculating the mean of the paired differences of each duplicate measurement divided by the mean of these measurements. This coefficient was calculated for each LPS concentration.

For the whole blood assay the coefficient of variation (the standard deviation (SD) divided by the mean) was determined for each LPS concentration. For this calculation the mean of the duplicate measurements were used. The coefficients of variation were calculated pair wise in repeated experiments (yielding the mean of individual coefficients of variation). Because the coefficient of variation is less subject to scaling effects as compared to the SD, values of the coefficient of variation for different experimental conditions were pooled to assess overall variation. Comparison of means was performed by ANOVA or its non-parametric alternative (Mann-Whitney *U* test) where appropriate. Furthermore, we performed an analysis of variance to estimate day-to-day and inter-individual variance, adjusted for LPS concentrations and the individual tested.

Calculations of the correlation coefficient between duplicate measurements were assessed non-parametrically using Spearman correlation test.

Results

Within ELISA variation.

For each of the LPS concentrations thirty-six duplicate ELISA measurements for TNF-α and thirty for IL-10 were available. The spread of the TNF-α and IL-10 ELISA

measurement was estimated as described in the methods. Briefly, the paired differences of each duplicate measurement were divided by their mean. For each LPS concentration the mean of these coefficients (eighteen for TNF-α and fifteen for IL-10) was calculated, revealing the mean spread relative to the mean for both TNF-α and IL-10 at each of the LPS concentrations. For TNF-α this mean spread relative to mean, after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, was 10.9, 11.9, 13.2, 11.9, 14.5 and 15.1%, respectively. Since these percentages were not statistically different, the data were pooled revealing an overall spread relative to the mean of 12.9%. For IL-10 the spread relative to the mean after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS was 37.5, 11.2, 17.0, 17.5, 19.1 and 13.6%, respectively. The percentage after stimulation with 0.1 ng/mL was high as compared to the other percentages, likely due to low concentrations of IL-10 (i.e. close to the level of detection) leading to a relatively high impact of even small differences in the test result (i.e. scaling effect). For this reason in the calculation of the overall spread relative to the mean, the data after stimulation with 0.1 ng/ml LPS were disregarded, leading to an overall spread relative to the mean of 15.7%.

TNF-α and IL-10 within whole blood stimulation variation.

The variation in the whole blood stimulation assay was investigated by performing the assay in duplicate; for each assay a single tube, drawn from one subject through a single vena puncture, was used as described in the methods. The cytokine measurements were performed on a single day, using a single ELISA batch. In each supernatant the cytokine measurement was performed in duplicate and the mean of the two ELISA measurements was used for this analyses.

The TNF-α concentration found with the first whole blood stimulation correlated with the second with r = 0.812, 0.676, 0.664, 0.773, 0.827 and 0.767 after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Correlation between the two TNF-α measurements after stimulation with all LPS concentrations combined was very high (r = 0.961). The same procedure was done for the IL-10 assay. The IL-10 concentration found with the first whole blood stimulation correlated with the second with r = 0.959, 0.946, 0.693, 0.782, 0.776, 0.979 after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Correlation between the two IL-10 measurements after stimulation with all LPS concentrations combined was very high (r = 0.979). A plot of the differences against the means, to estimate the repeatability of the assay, is given in Fig. 1 for TNF-α and Fig. 2 for IL-10.

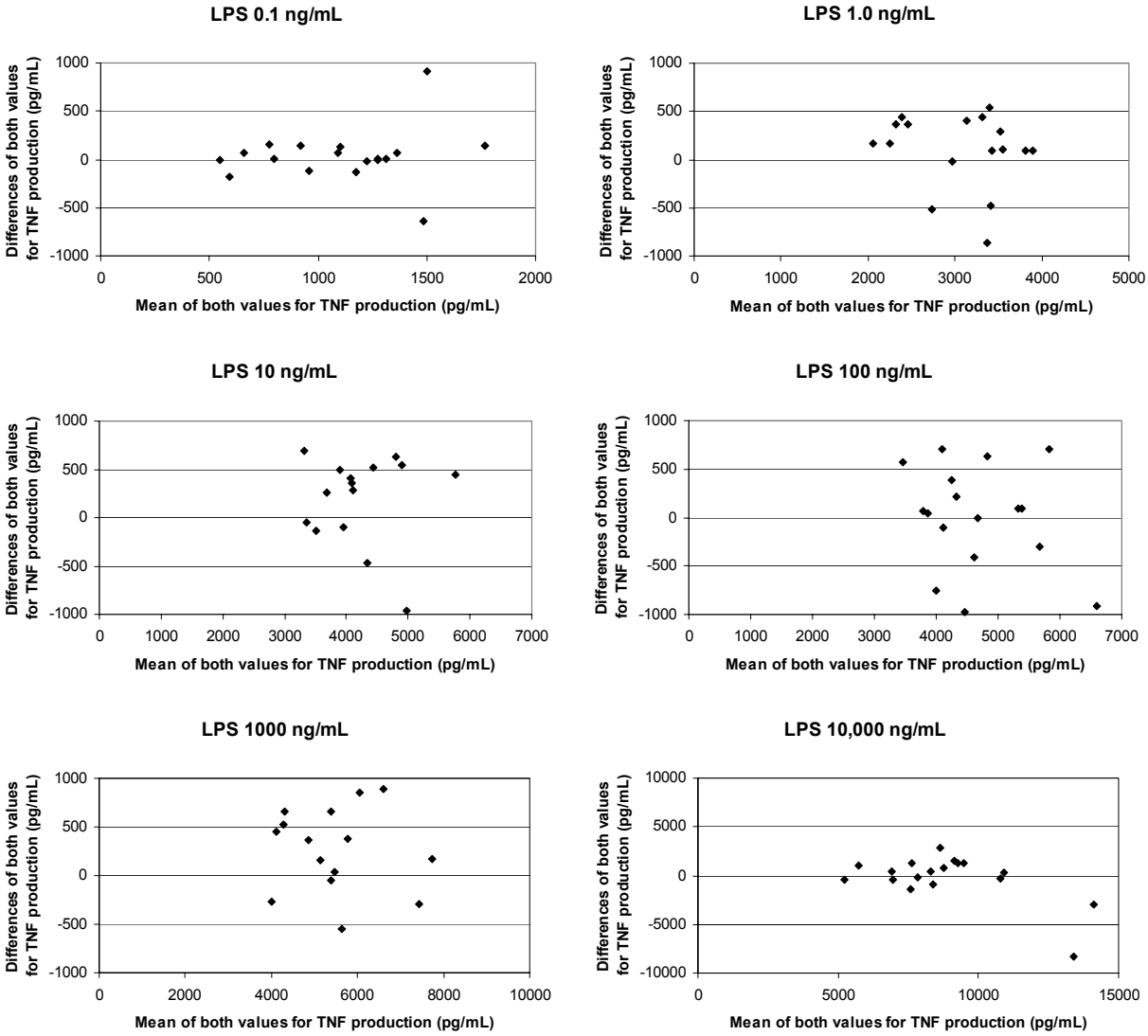

Figure 1. Ex vivo production of TNF-α in 18 whole blood samples in two subjects stimulated in duplicate with 0.1, 1, 10, 100, 1000 and 10.000 ng/mL lipopolysaccharide. Each dot represents the difference between both measurements (first minus second) dependent on the mean of both measurements.

For TNF-α the mean of the paired differences using all data was 17.2 pg/mL (95% confidence interval around the mean difference -2346 to 1367 pg/mL). The coefficient of variation (the standard deviation (SD) divided by the mean) was 8.4, 15.6, 10.1, 10.7, 3.2 and 11.1% after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Taking together this leads to an overall mean coefficient of variation of 9.9%. For IL-10 the mean of the paired differences using all data was 136.7 pg/mL (95% confidence interval around the mean difference -392 to 1610 pg/mL). The coefficient of variation (the standard deviation (SD) divided by the mean) was 30.5, 9.1, 3.0, 3.0, 3.7 and 2.0% after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Taking these percentages taking together, ignoring the data from the stimulation with 0.1 ng/mL LPS for previously mentioned reasons, the overall mean coefficient of variation is 4.2%.

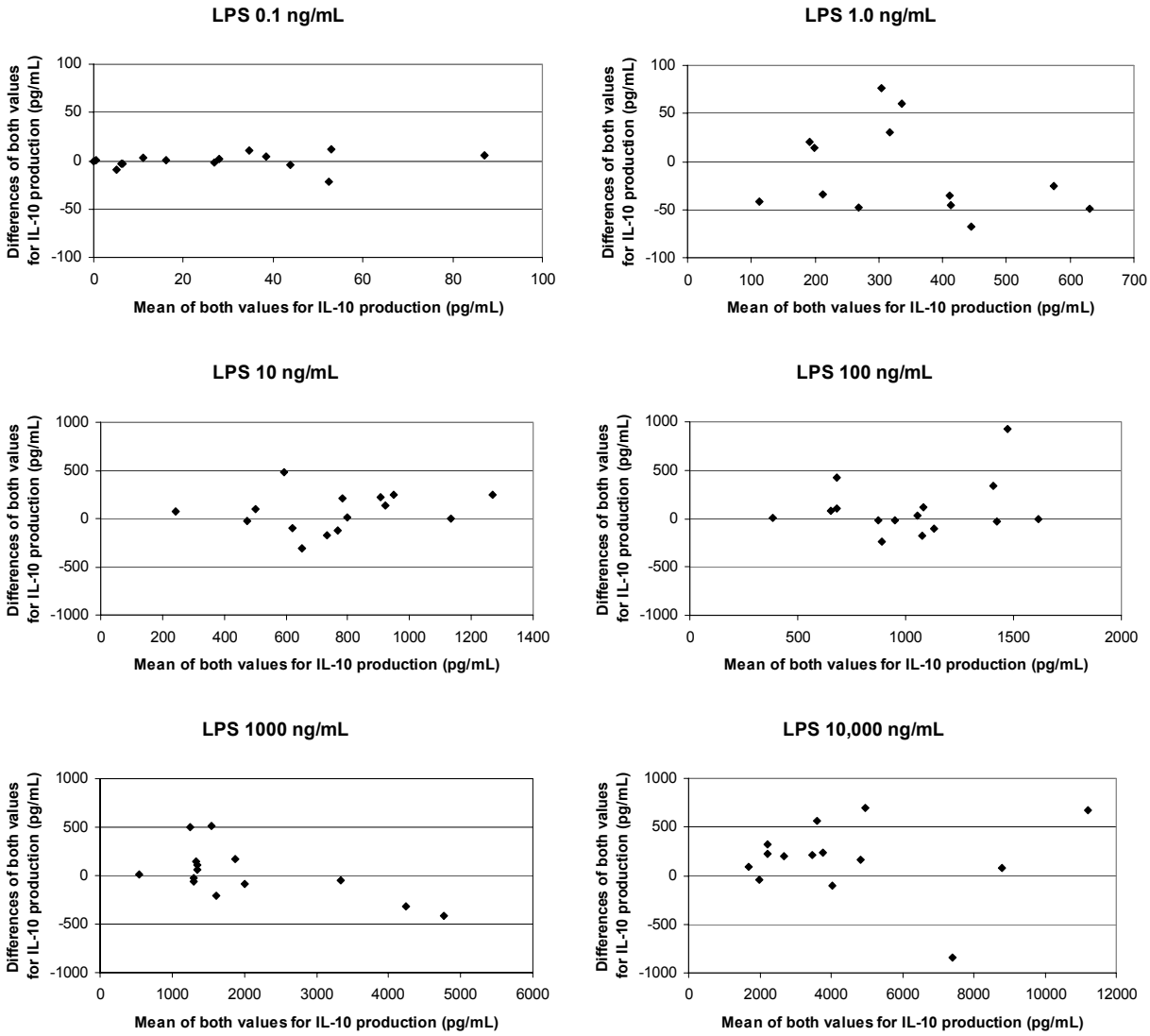

Figure 2. Ex vivo production of IL-10 in 15 whole blood samples in two subjects stimulated in duplicate with 0.1, 1, 10, 100, 1000 and 10.000 ng/mL lipopolysaccharide. Each dot represents the difference between both measurements (first minus second) dependent on the mean of both measurements.

Day-to-day variation in whole blood stimulation.

As described in the methods the whole blood stimulation was performed in two subjects over a period of three weeks. To investigate the day-to-day variation in the assay the mean cytokine concentration from two whole blood stimulations performed on the same day were used for each subject separately. The data are shown in Table 1. The coefficients of variation for TNF-α measurements were around 20% at each of the LPS concentrations in both subjects. For IL-10 the coefficients of variation were higher, i.e. around 35%. Coefficients for the IL-10 measurements were especially high in the extremes of the LPS concentrations used (Table 1).

Table 1. Intra-individual variation over 3 weeks in ex vivo production of TNF-α and IL-10 upon whole blood stimulation (performed in duplicate) with LPS in various concentrations

Subject	LPS (ng/mL)	TNF-α				IL-10			
		No. of observations	Mean (pg/mL)	SD (pg/mL)	CV (%)	No. of observations	Mean (pg/mL)	SD (pg/mL)	CV (%)
1	0.1	9	916	318	34.7	7	6.5	5.6	84.8
	1	9	2861	631	22.1	7	229	72	31.4
	10	9	4507	752	16.7	7	568	183	32.2
	100	9	4948	1096	22.2	7	822	332	40.4
	1000	9	5898	1513	25.7	7	1737	1180	67.9
	10,000	9	9293	2049	22.0	7	3895	1245	32.0
2	0.1	9	1286	256	19.9	8	45.5	19.5	42.9
	1	9	3282	422	12.9	8	502	116	23.1
	10	9	4106	544	13.2	8	921	206	22.4
	100	9	4598	776	16.9	8	1203	266	22.1
	1000	9	5524	1054	19.1	8	2652	1701	64.1
	10,000	9	8387	2617	31.2	8	5106	3555	69.6

Observations are means of two whole blood stimulation performed on a single day. CV, coefficient of variation expressing relative spread of ex vivo cytokine production over 3 weeks. LPS, lipopolysaccharide.

Multivariate analysis of variance.

Next, we examined in a multivariate model the variance in the TNF-α and IL-10 concentrations released within and between experiments. In this analysis TNF-α and IL-10 concentrations were analyzed after logtransformation. For TNF-α, there was a LPS dose-dependent ($p < 0.0001$) increase in the cytokine concentration measured. After adjusting for the LPS concentration, there was no significant difference in the concentration of TNF-α measured within separate runs (i.e. duplicate whole blood stimulations) performed on a single day; between days, the concentration of TNF-α measured differed significantly ($p = 0.005$), but this accounted for only about 4% of the total variance observed. Adjusting for the between day variance and LPS concentrations, the concentration of TNF-α measured did not differ between the two subjects. For IL-10, similarly to TNF-α, there was a LPS dose-dependent ($p < 0.0001$) increase in the cytokine concentration measured. After adjusting for the LPS concentration, there was no significant difference in the concentration of IL-10 measured within separate runs (i.e. duplicate whole blood stimulations) performed on single day; between days, the concentration of IL-10 measured differed somewhat, i.e., on average for about 2% of the total variance observed, but this inter-day difference did not reach a level of significance ($p = 0.066$). However, after

adjusting for the between day variance and LPS concentrations, the concentration of IL-10 measured did differ significantly between the subjects ($p < 0.001$), accounting for about 27% of the total between subjects variance observed.

Variation in dose-response characteristic using Hill-equation.

As described in the methods the results from the whole blood stimulation measurements using the wide range of LPS concentrations was used to calculate the dose-response characteristics using the receptor-ligand model, i.e. the E_{max} model using the Hill equation. For these calculations the means of the two whole blood stimulation assays performed on the same day were used (Table 1). The results are shown in Table 2.

Table 2. Intra-individual variation over 3 weeks of dose response characteristics calculated from TNF-α and IL-10 production upon whole blood stimulation (performed in duplicate) with LPS in various concentrations

Model	Charac-teristic	Subject 1				Subject 2				*p**
		No. of Observa-tions	Mean	SD	CV (%)	No. of Observa-tions	Mean	SD	CV (%)	
TNF-α										
1	EC_{50}	9	2.498	1.508	60.4	9	2.180	3.482	159.7	0.063
	E_{max}	9	6636	1416	21.3	9	6016	1614	26.8	0.489
2	EC_{50-1}	9	0.605	0.163	26.9	9	0.279	0.0924	33.1	<0.001
	E_{max-1}	9	4721	858.5	18.2	9	4297	615.3	14.3	0.340
	EC_{50-2}	9	$9.7x10^9$	$2.5x10^9$	257.7	9	4285	2420	56.5	0.605
	E_{max-2}	9	$3.2x10^9$	$8.4x10^9$	260.9	9	5808	3236	55.7	0.136
IL-10										
1	EC_{50}	7	1986	1135	57.2	8	721.5	839.6	116.4	0.029
	E_{max}	7	4630	1517	32.8	8	5418	4414	81.5	0.867
2	EC_{50-1}	7	2.017	0.805	39.9	8	1.090	0.439	40.3	0.014
	E_{max-1}	7	627.6	218.5	34.8	8	969.0	176.4	18.2	0.006
	EC_{50-2}	7	13629	20537	150.7	8	6204	11503	185.4	0.152
	E_{max-2}	7	6557	3036	46.3	8	6126	4830	78.8	0.779

Observations are means of two whole blood stimulation performed on a single day. CV, coefficient of variation expressing relative spread of ex vivo cytokine production over 3 weeks. LPS, lipopolysaccharide. EC_{50}, LPS concentration at which 50% of the maximal cytokine concentration is reached. E_{max}, maximal cytokine concentration. *Mann-Whitney *U* test.

For the TNF-α measurements nine measurements were available. As compared to model 1, model 2 revealed a good estimate of the EC_{50-1} as well as E_{max-1}. The coefficient of variation for TNF-α EC_{50-1} was 26.9 and 33.1 for subject 1 and 2, respectively. For the TNF-α E_{max-1}, these values were 18.2 and 14.3 for subject 1 and 2, respectively.

For IL-10, seven measurements were available for the first subject and eight were available for subject two. As for TNF-α model 2 performed best in a way that a rather precise estimation of EC_{50-1} as well as E_{max-1} could be made. The coefficient of variation for EC_{50-1} was 39.9 and 40.3 for subject 1 and 2, respectively. For the E_{max-1}, these values were 34.8 and 18.2, respectively (Table 2).

Finally for each of the subjects a best estimation of the dose response characteristics was calculated, using the same measurements as described above, for each of the cytokines. The results of this analysis are shown in Table 3.

Table 3. Dose response characteristics of TNF-α and IL-10 calculated from repeated whole blood stimulation assays with LPS in various concentrations in two subjects

Model	Characteristic	Subject 1		Subject 2	
		No. of observations	Value	No. of observations	Value
TNF-α					
1	EC_{50}	9	1.903	9	0.8816
	E_{max}	9	6565	9	5855
2	EC_{50-1}	9	0.6033	9	0.2613
	E_{max-1}	9	4780	9	4280
	EC_{50-2}	9	4981	9	3284
	E_{max-2}	9	6761	9	5452
IL-10					
1	EC_{50}	7	1279	8	829
	E_{max}	7	4350	8	5421
2	EC_{50-1}	7	2.212	8	1.014
	E_{max-1}	7	692.1	8	981.2
	EC_{50-2}	7	2961	8	1952
	E_{max-2}	7	4152	8	4932

Observations are means of two whole blood stimulation performed on a single day. CV, coefficient of variation expressing relative spread of ex vivo cytokine production over 3 weeks. LPS, lipopolysaccharide. EC_{50}, LPS concentration at which 50% of the maximal cytokine concentration is reached. E_{max}, maximal cytokine concentration.

Using these characteristics, the cytokine concentrations were calculated using model 1 and model 2 for each of the LPS concentrations used. The results for both subjects are shown in Fig. 3A and B (for TNF-α) and Fig. 3C and D (for IL-10).

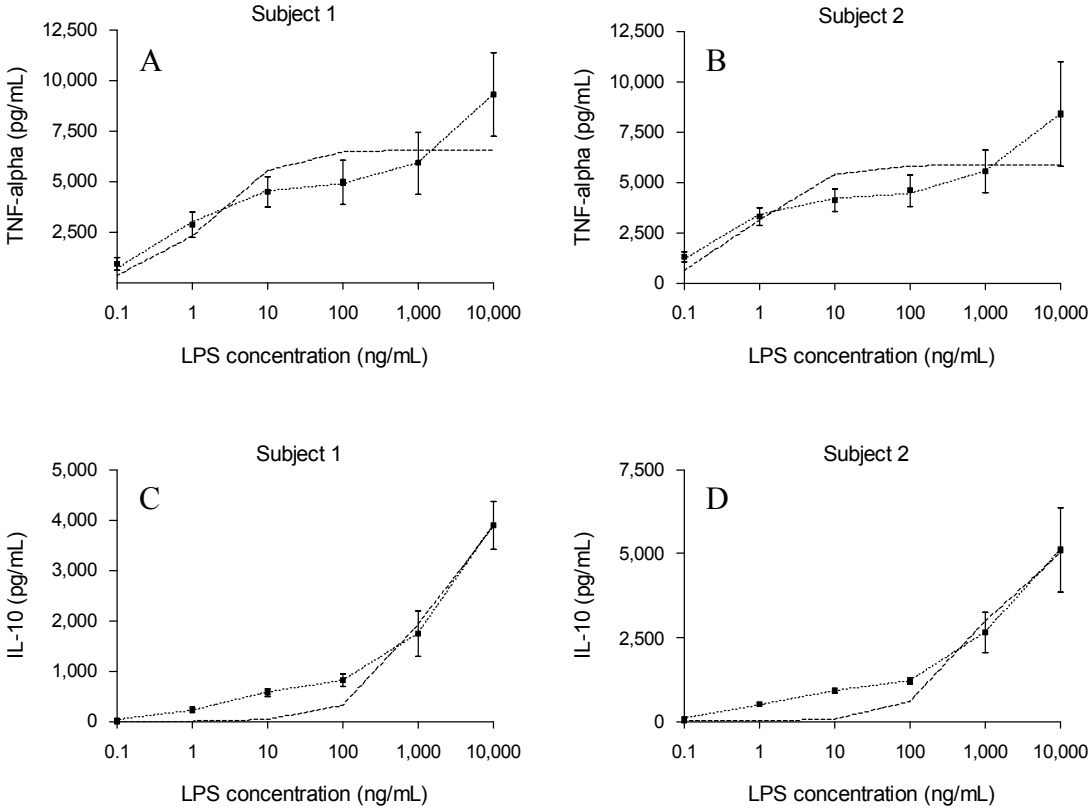

Figure 3. Ex vivo production of TNF-α in subject 1 (A) and subject 2 (B), IL-10 in subject 1 (C) and subject 2 (D). Whole blood stimulation was performed nine times over a 3 week period in duplicate. Each data point represents the mean of the two whole blood stimulations performed on the same day and the standard deviation (SD). Lines represent the fitted concentrations predicted by model 1 (---) and model 2 (…), respectively.

Comparison of TNF-α and IL-10 measurements between the subjects.

First, at each of the LPS concentrations the cytokine concentrations were compared between the two subjects. For TNF-α there was a significant difference in the concentrations found after stimulation with 0.1 ng/mL ($p = 0.019$, Mann-Whitney *U* test). After stimulation with the other LPS concentration no statistically difference differences between the two subjects were found (ANOVA and Mann-Whitney *U* test). Secondly, the dose response characteristics were compared between the two subjects. The results are shown in Table 2. Both with model 1 and model 2 the $EC_{50\text{-}1}$ values were higher in subject 1 as compared to subject 2, however the difference was only statistically different using model 2 ($p < 0.001$, Mann-Whitney *U* test, Table 2). For IL-10 the concentrations were different between the two subjects after stimulation with 0.1, 1, 10 and 100 ng/mL LPS ($p < 0.001$, <0.001, 0.004 and 0.021, Mann-Whitney *U* test, respectively, data not shown). The $EC_{50\text{-}1}$ values derived from model 1 and model 2 were statistically different between the two subjects ($p = 0.029$ and 0.014, respectively, Mann-Whitney U test). Furthermore, the $E_{max\text{-}1}$ derived from model 2 was statistically different between the two subjects ($p = 0.006$, Mann-Whitney *U* test, Table 2).

Discussion

The main finding of the present study is that the dose-response characteristics of TNF-α and IL-10 release by human peripheral blood cells, upon stimulation with a wide range of LPS concentrations, can be described adequately by a receptor-ligand interaction model that is fully characterized by two parameters, i.e., EC_{50}, the estimated LPS concentration at which half of the cytokine concentration is reached and the E_{max}, the estimated maximal concentration of cytokine released. Repeated measurements made over several weeks disclosed that these two parameters were highly constant for individuals, yet differed between individuals. These differences in dose-response characteristics would have been discarded however, if only one or two LPS concentrations had been used to test the cytokine response of these individuals. For instance, only the TNF-α concentration measured after stimulation with the lowest LPS concentration (0.1 ng/mL) was different between the subjects, whereas IL-10 concentrations were only different between the subjects after stimulation with the lower LPS concentrations (i.e. 0.1 through 100 ng/mL). These findings suggest that relevant information on cytokine release is lost, when the commonly applied approach is taken, i.e. testing the TNF-α and or IL-10 release after stimulation with a single, and often high, LPS concentration. Such an approach yields one value that doesn't represent a physiological model of release and often shows variation over time. By contrast, we estimated two parameters that fully characterize an underlying model and are intrinsic parameters that are insensitive to day-to-day variation.

Two examples may illustrate the relevance of our approach. First, the TNF-α-308 AA or GA genotypes are generally being termed as the high TNF-α producing genotypes and the TNF-α-308 GG as the low producing genotype. A study using reporter gene constructs demonstrated convincingly that the -308A allele is a much stronger transcriptional activator as compared to the more frequently occurring -308G allele (13). However, studies using ex vivo stimulation assays using various stimuli, cells and incubation times revealed conflicting results (8;14;17;18;20;23;25-29;32). In one study a difference between the two TNF-308 genotypes was detected at the lowest LPS concentration (1 ng/mL), whereas at the high concentration (100 ng/mL), no difference was detected (8). Although comparison of the studies is hampered by differences in the assays used, studies using low LPS concentrations or other rather poor stimuli are more likely to detect (non-significant) differences between the two TNF-308 genotypes (17;18;26). Although we did not determine genetic factors in our study, we detected significant differences between the two subjects at the lowest LPS concentration used, and more importantly the dose-response parameters based on the whole range of LPS concentrations remained significantly different between the subjects.

Secondly, The IL-10-1082 GG and GA genotypes are generally termed as high IL-10 producing and the IL-10-1082 AA as the low producing genotype. In a previous study in cardio-thoracic surgery patients, we were only able to confirm this finding in carriers of the GATA allele after stimulation with low LPS concentrations (10 and 100 ng/mL), whereas no differences were detected at the highest LPS concentrations (1000 ng/mL) (30). More interestingly the EC_{50} value, estimated from the measurements of IL-10 after stimulation with the whole range of LPS concentrations, was significantly higher in carriers of the GATA haplotype, indicating lower LPS sensitivity. Furthermore, carriers of the AGCC allele were only distinguishable from the other individuals based on the estimated EC_{50}, whereas IL-10 concentrations produced upon stimulation with a range of LPS concentrations (10, 100 and 1000 ng/mL LPS) were not different. Similarly to this example, we expect that discrepancies found in studies results could well be explained by differences in the LPS stimulus used in the ex vivo assay. Either the concentration or the composition of the LPS used could be different in the way that the cytokine concentrations measured are at different levels of the dose-response curve. Our approach could to some extend normalize these differences. By calculating the dose-response characteristic based on a wide range of LPS concentrations the intrinsic dose-response characteristics could be calculated, making comparison between studies possible.

The whole blood stimulation we used is similar to the assays used by others. In a previous study similar variation coefficients were found in repeated whole blood stimulation measurements over a smaller LPS range as used in our study. Similar to our results this study showed slightly higher variation in IL-10 production as compared to TNF-α. Due to the small range of LPS concentrations used in this study (ranging from 10-1000 ng/mL) calculation of dose-response characteristics was not possible.

So far, no other studies used the approach of estimating dose-response characteristics for an individual based on cytokine production upon a wide range of LPS concentrations in a whole blood stimulation system. In previous studies, based on the observed cytokine production ex vivo, individuals were designated as 'high' or 'low' producers. Also, individuals with specific polymorphisms of their cytokine genes, were given the same labels, after the presumed relationship between that genotype and the corresponding in vitro cytokine production profile.

In summary, we conclude that labeling subjects as ‘high’ or ‘low’ cytokine producers based on the in vitro production of cytokines after stimulation with one single, supraphysiological LPS concentration, might proof to be to rough a measure to describe a subjects cytokine production profile. Based on our result we expect that characterization of an individual's cytokine production profile by means of dose-response characteristics, calculated from in vitro cytokine production upon a wide range of LPS concentrations, is a

more suitable approach. Studies aimed at correlating in vitro with in vivo phenotypes and genetic polymorphisms should for this purpose use large dose-response ranges of whole blood stimulation with LPS to be able to closely estimate these dose-response characteristics. These dose-response characteristics are more likely to be good estimates of an individual's in vitro cytokine production profile and therefore more suitable as a reference used in studies that investigate the genetic factors that make up for the large inter-individual variation found in cytokine production upon whole blood stimulation with LPS.

Reference List

(1) van der Linden MW, Huizinga TW, Stoeken DJ, Sturk A, Westendorp RG. Determination of tumour necrosis factor-alpha and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. J Immunol Methods 1998; 218(1-2):63-71.

(2) Westendorp RG, Langermans JA, Huizinga TW, Elouali AH, Verweij CL, Boomsma DI et al. Genetic influence on cytokine production and fatal meningococcal disease. Lancet 1997; 349(9046):170-173.

(3) Reuss E, Fimmers R, Kruger A, Becker C, Rittner C, Hohler T. Differential regulation of interleukin-10 production by genetic and environmental factors--a twin study. Genes Immun 2002; 3(7):407-413.

(4) Eskdale J, Gallagher G, Verweij CL, Keijsers V, Westendorp RG, Huizinga TW. Interleukin 10 secretion in relation to human IL-10 locus haplotypes. Proc Natl Acad Sci U S A 1998; 95(16):9465-9470.

(5) de Jong BA, Westendorp RG, Bakker AM, Huizinga TW. Polymorphisms in or near tumour necrosis factor (TNF)-gene do not determine levels of endotoxin-induced TNF production. Genes Immun 2002; 3(1):25-29.

(6) Gibson AW, Edberg JC, Wu J, Westendorp RG, Huizinga TW, Kimberly RP. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. J Immunol 2001; 166(6):3915-3922.

(7) Huizinga TW, Keijsers V, Yanni G, Hall M, Ramage W, Lanchbury J et al. Are differences in interleukin 10 production associated with joint damage? Rheumatology (Oxford) 2000; 39(11):1180-1188.

(8) Louis E, Franchimont D, Piron A, Gevaert Y, Schaaf-Lafontaine N, Roland S et al. Tumour necrosis factor (TNF) gene polymorphism influences TNF-alpha production in lipopolysaccharide (LPS)-stimulated whole blood cell culture in healthy humans. Clin Exp Immunol 1998; 113(3):401-406.

(9) Koss K, Satsangi J, Fanning GC, Welsh KI, Jewell DP. Cytokine (TNF alpha, LT alpha and IL-10) polymorphisms in inflammatory bowel diseases and normal controls: differential effects on production and allele frequencies. Genes Immun 2000; 1(3):185-190.

(10) Cuenca J, Cuchacovich M, Perez C, Ferreira L, Aguirre A, Schiattino I et al. The -308 polymorphism in the tumour necrosis factor (TNF) gene promoter region and ex vivo lipopolysaccharide-induced TNF expression and cytotoxic activity in Chilean patients with rheumatoid arthritis. Rheumatology (Oxford) 2003; 42(2):308-313.

(11) Stuber F, Udalova IA, Book M, Drutskaya LN, Kuprash DV, Turetskaya RL et al. -308 tumor necrosis factor (TNF) polymorphism is not associated with survival in severe sepsis and is unrelated to lipopolysaccharide inducibility of the human TNF promoter. J Inflamm 1995; 46(1):42-50.

(12) Kroeger KM, Carville KS, Abraham LJ. The -308 tumor necrosis factor-alpha promoter polymorphism effects transcription. Mol Immunol 1997; 34(5):391-399.

(13) Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci U S A 1997; 94(7):3195-3199.

(14) Schippers EF, van 't Veer C, van Voorden S, Martina CA, le Cessie S, van Dissel JT. TNF-alpha promoter, Nod2 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin. Cytokine 2004; 26(1):16-24.

(15) Heesen M, Schippers EF, Bloemeke B, Kunz D, van Dissel JT. Cytokine response to endotoxin in individuals heterozygous for the Delta32 mutation of chemokine receptor CCR5. Cytokine 2003; 21(4):195-199.

(16) Heesen M, Blomeke B, Schluter B, Heussen N, Rossaint R, Kunz D. Lack of association between the -260 C-->T promoter polymorphism of the endotoxin receptor CD14 gene and the CD14 density of unstimulated human monocytes and soluble CD14 plasma levels. Intensive Care Med 2001; 27(11):1770-1775.

(17) Huizinga TW, Westendorp RG, Bollen EL, Keijsers V, Brinkman BM, Langermans JA et al. TNF-alpha promoter polymorphisms, production and susceptibility to multiple sclerosis in different groups of patients. J Neuroimmunol 1997; 72(2):149-153.

(18) Pociot F, Wilson AG, Nerup J, Duff GW. No independent association between a tumor necrosis factor-alpha promotor region polymorphism and insulin-dependent diabetes mellitus. Eur J Immunol 1993; 23(11):3050-3053.

(19) Turner D, Grant SC, Yonan N, Sheldon S, Dyer PA, Sinnott PJ et al. Cytokine gene polymorphism and heart transplant rejection. Transplantation 1997; 64(5):776-779.

(20) Mycko M, Kowalski W, Kwinkowski M, Buenafe AC, Szymanska B, Tronczynska E et al. Multiple sclerosis: the frequency of allelic forms of tumor necrosis factor and lymphotoxin-alpha. J Neuroimmunol 1998; 84(2):198-206.

(21) Crawley E, Kay R, Sillibourne J, Patel P, Hutchinson I, Woo P. Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. Arthritis Rheum 1999; 42(6):1101-1108.

(22) Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 1997; 24(1):1-8.

(23) Hutchings A, Guay-Woodford L, Thomas JM, Young CJ, Purcell WM, Pravica V et al. Association of cytokine single nucleotide polymorphisms with B7 costimulatory molecules in kidney allograft recipients. Pediatr Transplant 2002; 6(1):69-77.

(24) Warle MC, Farhan A, Metselaar HJ, Hop WC, Perrey C, Zondervan PE et al. Are cytokine gene polymorphisms related to in vitro cytokine production profiles? Liver Transpl 2003; 9(2):170-181.

(25) Danis VA, Millington M, Hyland V, Lawford R, Huang Q, Grennan D. Increased frequency of the uncommon allele of a tumour necrosis factor alpha gene polymorphism in rheumatoid arthritis and systemic lupus erythematosus. Dis Markers 1995; 12(2):127-133.

(26) Bouma G, Crusius JB, Oudkerk PM, Kolkman JJ, von Blomberg BM, Kostense PJ et al. Secretion of tumour necrosis factor alpha and lymphotoxin alpha in relation to polymorphisms in the TNF genes and HLA-DR alleles. Relevance for inflammatory bowel disease. Scand J Immunol 1996; 43(4):456-463.

(27) Knuchel MC, Spira TJ, Neumann AU, Xiao L, Rudolph DL, Phair J et al. Analysis of a biallelic polymorphism in the tumor necrosis factor alpha promoter and HIV type 1 disease progression. AIDS Res Hum Retroviruses 1998; 14(4):305-309.

(28) Somoskovi A, Zissel G, Seitzer U, Gerdes J, Schlaak M, Muller Q. Polymorphisms at position -308 in the promoter region of the TNF-alpha and in the first intron of the TNF-beta genes and spontaneous and lipopolysaccharide-induced TNF-alpha release in sarcoidosis. Cytokine 1999; 11(11):882-887.

(29) Hoffmann SC, Stanley EM, Darrin CE, Craighead N, DiMercurio BS, Koziol DE et al. Association of cytokine polymorphic inheritance and in vitro cytokine production in anti-CD3/CD28-stimulated peripheral blood lymphocytes. Transplantation 2001; 72(8):1444-1450.

(30) Schippers EF, van 't Veer C, van Voorden JB, Huizinga T, le Cessie S, van Dissel JT. IL-10 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin. Cytokine 2005; 29(5):215-228.

(31) Holford NH, Sheiner LB. Understanding the dose-effect relationship: clinical application of pharmacokinetic-pharmacodynamic models. Clin Pharmacokinet 1981; 6(6):429-453.

(32) Turner DM, Grant SC, Lamb WR, Brenchley PE, Dyer PA, Sinnott PJ et al. A genetic marker of high TNF-alpha production in heart transplant recipients. Transplantation 1995; 60(10):1113-1117.