CHAPTER 3

PERFORMANCE OF THE WHOLE-BLOOD STIMULATION ASSAY FOR ASSESSING INNATE IMMUNE ACTIVATION UNDER FIELD CONDITIONS

Linda May
David van Bodegom
Maris Kuningas
Hans J Meij
Anton JM de Craen
Marijke Frölich
Rudi GJ Westendorp

Cytokine 2009; 45(3): 184-189
ABSTRACT

Innate propensity of immune activation is reflected in production of pro- and anti-inflammatory cytokines upon stimulation of Toll-like receptors (TLR) in whole-blood stimulation assays. The validity of the whole-blood stimulation assay under field conditions has not been evaluated extensively. Here, we have determined correlation of individually repeated whole-blood stimulation assays in a field-study in Ghana and compared it with that of two Dutch populations performed under optimal conditions. We also examined cytokine production to various TLR-agonists in order to create an assay that would mimic general innate immune responses. Under field conditions repeated assessments of lipopolysaccharide- induced Tumor Necrosis Factor-α (TNFα) production were poorly correlated (r=0.15, p=0.087). Correlation was relatively high for production of Interleukin-10 (IL10) (r=0.48, p<0.001) and comparable to that observed in the Dutch population under optimal conditions. Combined stimulation with lipopolysaccharide and zymosan resulted in cytokine production profiles that were similar to that attained after stimulation with a mixed culture of bacteria. Here, we conclude that variation of a whole-blood assay performed in field setting is large in general but that production of IL10 seems to better reflect an innate pro- or anti-inflammatory tendency whereas production of TNFα may predominantly reflect recent immunological challenges. Furthermore, simultaneous stimulation of several Toll-like receptors may mimic general innate immune activation.
INTRODUCTION

Cytokines are key regulators of the innate immune response. For assessment of an individual's production of pro- and anti-inflammatory cytokines, a whole-blood stimulation assay has been developed\(^1\). Earlier data on the performance of the assay under optimal conditions have shown that production of cytokines is highly reproducible\(^2\) and under strict genetic control\(^3\). Yet the assay would be extremely useful for studying cytokine production in epidemiological setting in developing countries, for example to study innate immune responses towards infection\(^4,5\). Under field conditions the question is whether additional laboratory variation introduced due to limited equipment and adapted techniques, will result in extra variation of the whole-blood stimulation assay. In addition, participants in a developing country have higher exposure to pathogens and fluctuations in nutritional status that might influence the cytokine responsiveness to a larger extent compared to populations living under affluent conditions. Depending on the research question, it is quite essential to identify whether cytokine production profiles obtained with the whole blood stimulation assay predominantly reflect recent immunological challenges rather than being consistent over time and more likely to reflect a genetically determined propensity.

Up to date most whole-blood stimulation assays have made use of *Escherichia coli* lipopolysaccharide (LPS), a ligand of the Toll-like receptor-4 (TLR4)\(^6\). It has been argued that TLR4 stimulated cytokine production is informative on innate immune activation upon gram-negative infections\(^7\), but inconclusive for gaining knowledge on innate immune activation in a broad sense. Such a broad read-out of the innate immune response is necessary when studying life-history characteristics such as aging\(^8,9\) and fertility\(^10\), but also for studying risk of autoimmune diseases\(^11\) and cardiovascular diseases\(^12\). From another angle, but arguing the same principle, it is questioned whether stimulation of a single Toll-like receptor is comprehensive to study innate immune responsiveness to infection, or that other Toll-like receptors would be of additive value\(^13-17\). As *in vivo* several pathways are involved in cytokine regulation the best stimulation *ex vivo* would be obtained by stimulating several Toll-like receptors simultaneously.

To validate the whole-blood stimulation assay for the use under field conditions, we first compared the correlation of repeated measurements in a
Ghanaian population with that in two Dutch populations. Furthermore to develop a whole-blood stimulation assay that best reflects general cytokine production in a broader sense, we performed a series of whole-blood stimulation assays using various TLR-agonists in addition to an assay that accidentally was contaminated with a mix of bacteria.

**MATERIALS & METHODS**

*Study population and research facilities*

Part of this study was conducted in the Garu-Tempane district, Upper East Region, Ghana. The region is rural and has one rainy season per year. The inhabitants are poor and belong to different ethnic backgrounds, mostly Bimoba (66%) and Kusasi (27%). In the study area two field visits were performed, in July/August 2005 and July/August 2006. During these visits healthy people were approached to volunteer for blood collection. In total 696 people were included in 2005 and 663 in 2006. Especially for this study a field-laboratory was improvised that was suited for whole-blood stimulation assays only. Ethical approval was given by the Leiden University Medical Center, The Netherlands, the district health officers of the Upper East Region and by the Ghanaian Ministry of Health, Medical Ethical Committee. Informed consent was obtained from all people included in the research.

*Additional populations*

For comparisons, whole-blood stimulated cytokine data from two Dutch populations were used. These included 492 participants from the “Leiden 85 plus Study” (a prospective cohort-study of elderly inhabitants of Leiden, the Netherlands\(^1\)\(^8\)), and 389 participants from the “Prosper study” (a prospective multicenter, randomized, placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly individuals\(^1\)\(^9\)). Whole-blood stimulation assays were performed in The Netherlands in a well-equipped laboratory, using standard methods as described elsewhere\(^2\). At two time-points LPS-induced cytokines were measured; at age 85 and age 86 for the first population and before and three months after treatment with pravastatin for the second population.
Whole-blood stimulation assays in Ghana

Four millilitres venous blood was collected in the morning in a sterile endotoxin-free lithium heparin tube (Greiner BioOne GmbH, Austria) and was suspended 1:1 with RPMI-1640 (with 25 mM Hepes and L-glutamine, Gibco, Breda, The Netherlands). The first series were incubated in presence and absence of 10 µg/ml *E. coli* LPS (a TLR4 ligand) (0111:B4 L2630, phenol extracted, Sigma Aldrich, Zwijndrecht, The Netherlands). Samples without LPS were control samples for TNFα background. In the second series besides 10 µg/ml LPS, also 100 µg/ml *S. cerevisiae* zymosan A (a TLR2 ligand) (Sigma Z4250, Schnelldorf, Germany) and co-stimulation of 10 ng/ml LPS and 100 µg/ml zymosan was used. In that series, the incubation medium was supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin (15140-122, Invitrogen, Breda, The Netherlands) to avoid bacterial contamination. Pre-testing of antibiotics revealed no differences in cytokine production (data not shown). Cytokine enriched supernatants were obtained after culturing blood for 24h at 37°C in a humidiﬁed atmosphere containing CO₂. As no CO₂ tank was available, to create this atmosphere samples were put in a tightly closed plastic container, containing water and a burning candle. Pre-testing of this method revealed high correlation of cytokine production after the candle method and the original CO₂ stove method (data not shown). Supernatants were kept at -20°C and transported on dry ice to the Netherlands.

Cytokine assays

TNFα and IL10 cytokine concentrations of all study populations were measured in the Netherlands by Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer’s guidelines (PeliKine Compact™ human TNFα and IL10, Sanquin Reagents, Amsterdam, The Netherlands).

Bacterial contamination

TNFα background higher than 100 pg/ml in control samples was used as a cut-off for bacterial contamination. Out of the 696 samples of the first series, 185 were above this cut-off, of which 59% even reached far beyond the upper detection limit. Three randomly taken samples with high background showed contamination with a mix of Gram-positive rods, chains and cocci among which enterococci and staphylococci, as well as Gram-negative rods ranging from 1000 to >100,000 SFU/gram. Four randomly taken samples with a background lower than 100 pg/ml were all negative for bacteria. Bacterial contamination had occurred during a limited time period in the
beginning of the first field visit after which contamination had disappeared completely.

**Statistical analyzes**

As cytokine levels were non-normally distributed log-transformed data was used for assessment of the agreement of two series of whole-blood assays by the Pearson correlation coefficient. To assess the differences in cytokine production to various TLR-agonists within one assay T-tests were used. Cytokine production was further presented as geometric means with corresponding standard errors. Since absolute values of cytokine production were different between the first and the second measurement, log-transformed data were transformed to z-scores to make visual comparisons of cytokine production in two series and to various stimuli. All analyzes were performed with SPSS 14.0 statistical software (SPSS Inc., Chicago, IL, USA).

**RESULTS**

Table 1 provides the characteristics and number of whole-blood stimulation assays, and the number of repeated measurements among the Ghanaian population under field conditions and two series of measurements among the Dutch population under optimal conditions.

<table>
<thead>
<tr>
<th>Population</th>
<th>Stimulus</th>
<th>Number per series</th>
<th>TLRs involved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>Ghanaian</td>
<td>LPS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>511</td>
<td>663</td>
</tr>
<tr>
<td></td>
<td>Live bacteria&lt;sup&gt;b&lt;/sup&gt;</td>
<td>185</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Zymosan&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>662</td>
</tr>
<tr>
<td></td>
<td>LPS&lt;sup&gt;a&lt;/sup&gt; + zymosan&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>657</td>
</tr>
<tr>
<td>Dutch population (“85 plus”)</td>
<td>LPS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>561</td>
<td>493</td>
</tr>
<tr>
<td>Dutch population (“Prosper”)</td>
<td>LPS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>403</td>
<td>392</td>
</tr>
</tbody>
</table>

<sup>a</sup> *E. coli* lipopolysaccharide  
<sup>b</sup> LPS stimulated, contaminated with a mix of Gram-positive rods, chains and cocci and Gram-negative rods ranging from 1,000 to >100,000 SFU/gram  
<sup>c</sup> *S. cerevisiae* zymosan
Correlation of repeated measurements

Figure 1 shows the correlation of cytokine production in two series of whole-blood stimulation assays. Cytokine production of TNFα and IL10 upon stimulation with LPS was measured twice in 127 individuals with an interval of one year. As absolute levels of cytokines were different in the two series, these were plotted as deviation from the sample mean (z-scores). The figure shows that TNFα levels are less correlated (r=0.15, p=0.087) than IL10 (r=0.48, p<0.001), especially when the values were low. In the two Dutch studies the correlations ranged from r=0.55 (p<0.001) to r=0.60 (p<0.001) for TNFα and from r=0.62 (p<0.001) to r=0.77 (p<0.001) for IL10 (Table 2). The repeatability of TNFα/IL10 was r=0.306, p=0.001 for Ghana, r=0.451 (p<0.001) for the first Dutch population (Leiden 85+) and r=0.604 (p<0.001) for the second Dutch population (“Prosper”).

Figure 1. Correlation of repeated cytokine production by ex vivo LPS-stimulated whole-blood stimulation assays performed in a field-based laboratory. From 127 Ghanaian individuals whole-blood was stimulated with E. coli LPS at two occasions with an interval of one year. Cytokine production is presented as deviations from the population mean (z-scores).
**Choice of TLR-agonist**

To compare cytokine production patterns to various TLR-agonists we made use of a contaminated series of whole-blood stimulation assays, which mimics the stimulation with whole bacteria. TNFα production after stimulation with LPS (geometric mean [±1SE], 5.06 [4.87–5.27] ng/ml) and whole bacteria (4.57 [4.18–4.99] ng/ml), was not different (p=0.289). In contrast, IL10 production was lower after stimulation in the contaminated series (0.31 [0.27–0.36] ng/ml) (p<0.001) when compared to LPS-stimulation only (1.82 [1.75–1.89] ng/ml) (Figure 2 panel A). In the second series (Figure 2 panel B) LPS-induced TNFα production (12.30 [11.99–12.62] ng/ml) and zymosan-induced TNFα production (12.52 [12.22–12.83] ng/ml) were similar (p=0.484), but LPS and zymosan co-stimulation (12.99 [12.66 13.34] ng/ml) induced more TNFα when compared to zymosan alone (p=0.107), or when compared to LPS alone (p=0.037). In contrast, when compared to LPS-induced IL10 production (4.21 [4.12–4.29] ng/ml), zymosan-induced IL10 (0.23 [0.22–0.23] ng/ml, p<0.001) and LPS and zymosan co-stimulated IL10 production (0.61 [0.59–0.63] ng/ml, p<0.001) were lower. IL10 production of this co-stimulation was higher than that from stimulation with zymosan alone (p<0.001). Correlation of cytokine production induced by the two TLR-agonists LPS and zymosan was r=0.55 (p<0.001) for TNFα and r=0.59 (p<0.001) for IL10.

---

**Table 2. Correlations of repeated assessments of *ex vivo* TNFα and IL10 production by whole-blood stimulation assays under field conditions and optimal conditions.**

<table>
<thead>
<tr>
<th>cytokine</th>
<th>population source</th>
<th>n</th>
<th>interval (months)</th>
<th>correlation (r)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>Ghanaian population</td>
<td>127</td>
<td>12</td>
<td>0.15</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>Dutch population (“85 plus”)</td>
<td>492</td>
<td>12</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Dutch population (“Prosper”)</td>
<td>389</td>
<td>4</td>
<td>0.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL10</td>
<td>Ghanaian population</td>
<td>127</td>
<td>12</td>
<td>0.48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Dutch population (“85 plus”)</td>
<td>492</td>
<td>12</td>
<td>0.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Dutch population (“Prosper”)</td>
<td>389</td>
<td>4</td>
<td>0.77</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Blood samples were stimulated for 24 hours with 10μg/ml *E. coli* LPS in two series of whole-blood stimulation assays.
production (Figure 3). We also tested the correlation for stimulation with whole bacteria in the first series versus LPS/zymosan-stimulated cultures in the second series (n=62). These analyzes revealed a moderate correlation for IL10 levels (r=0.311, p=0.014), but no correlation for TNFα levels (r=0.007, p=0.956), when we adjusted for TNFα back-ground from the contaminated series, since this might reflect the amount of contamination present, this resulted in a correlation of r=0.104, p=0.727 for TNFα and r=0.512, p<0.001 for IL10.

![Cytokine production of whole-blood stimulation assays with different stimulations](image)

**Figure 2. Cytokine production of whole-blood stimulation assays with different stimulations.** In a first series (panel A) cytokines were induced by 24h stimulation with *E. Coli* LPS. Part of this series was contaminated with a mix of live Gram-positive and Gram-negative bacteria. In the second series (panel B) cytokine production was induced by several combinations of TLR-agonists: *E. coli* LPS, with *S. cerevisiae* zymosan, and with a co-stimulation of LPS and zymosan with the stimulations being performed in parallel in the same blood samples. Cytokine production is presented as geometric means with standard errors. *p=0.037, **p<0.001.
Chapter 3

Figure 3. Correlation of cytokine production from LPS-stimulated and zymosan-stimulated whole-blood stimulation assays. Cytokines were induced by \textit{E. coli} LPS and \textit{S. cerevisiae} zymosan in parallel in the same blood samples (n=662). Cytokine production is presented as deviations from the population mean (z-scores).

DISCUSSION

In this study we evaluated the performance of the whole-blood stimulation assay for the use under field conditions. The first aim was to assess whether cytokine production from a Ghanaian field-study was reproducible and was compared to that examined in two Dutch study populations under optimal conditions. The second aim was to develop an assay that was suited for assessing innate cytokine production patterns in a broad sense, an idea inspired by a contaminated series of whole-blood stimulation assays followed by tailor-made experiments.

Besides that in Ghana there was in general more variation in the whole-blood assay than in the Netherlands, from the cytokines TNF$\alpha$ production was more variable than IL10 production. In other studies, performed in well-equipped laboratories, TNF$\alpha$ also tends to give higher variation than IL10\textsuperscript{2,3,21}. In addition, variation in IL10 in Ghana was found similar as that of two Dutch study populations, whereas variation of TNF$\alpha$ was much larger than that of the Dutch studies. As all the cytokine ELISAs were performed in the same well-equipped clinical laboratory, one can argue that differences
in variation between the populations can be attributed to the study subjects, climate, the laboratorial practice during incubation or a combination of these. As fieldwork is performed in the rainy season in northern Ghana, infectious diseases, especially malaria, are endemic, which might cause additional variability in cytokine production. The same holds for nutrition status; food shortage is common in this population and is even at its maximum just before the harvest season, i.e. the period that this study was performed. It could be that genetic tendency towards a pro-or anti-inflammatory cytokine production pattern, as was earlier suggested, would only hold in a consistent environment. Despite that we tried to keep all conditions as consistent as possible, still a small amount of variation was probably introduced in this study. Furthermore, as our study was of longitudinal nature it cannot be excluded that the variation observed is related to immunological changes in the study population and not to differences in the performance of the whole-blood assay. Since in Ghana the study participants have probably had more immunological challenges than the Dutch, this could mask the genetic regulation of cytokine production. This seems to be the case for TNFα production which reflects more the environmental exposure. IL10 production on the other hand seems to reflect more the innate tendency towards a pro- or an anti-inflammatory cytokine production pattern, as its production is consistently maintained when performed under field conditions and comparable to that observed in a well-equipped laboratory. One might argue that the addition of antibiotics in only one of the series made the assays not completely alike. Indeed this may result in extra variation, however it has to be noted that this is a widely used method and has been pre-tested and found to be comparable to the assay without antibiotics.

In this study, we found confirmation to the notion that the choice of ligands in the whole-blood stimulation assay influence cytokine production. In different stimulations TNFα production was equal, whereas IL10 was lower in the contaminated samples or samples with zymosan, compared to LPS-stimulation. Furthermore, within one assay cytokine production after LPS or zymosan stimulation was corresponding only modestly. Different cytokine production per induced receptor could be due to different endotoxic potency of the ligands. LPS and zymosan induce different TLRs and pathways downstream; LPS induces the MyD88 dependent and independent pathway via TLR4, whereas zymosan induces predominantly the MyD88 dependent pathway via TLR2 in heterodimer with TLR6. Zymosan also uses Dectin-1...
as pattern-recognition receptor which has been suggested to block the IL10 receptor\textsuperscript{26-28}, resulting in lower production of IL10 in our sample. Low correspondence of individual tendency towards a pro- or an anti-inflammatory response for the different TLR-agonists might therefore be due to individual genetic variation in molecules that are involved in the several pathways downstream. This might result in selective hypo- or hyper-responsiveness to single TLR-agonists\textsuperscript{29}. Furthermore in real-life various infections do occur and immune activation can be in several directions and on various pathogen recognition receptors. One can imagine that in \textit{ex vivo} studies, where the usual practice is to stimulate with a single TLR-agonist, general immune regulation can not be captured and inconclusive results might be obtained. Therefore an ideal assay with regard to giving insight in a general innate immune response would induce more pathways with a mix of ligands. As the use of whole bacteria gives many practical drawbacks, we tried to incorporate stimuli, that would mimic those from the environment. The results of this study suggest that an assay with both TLR4 and TLR2 ligands is a good alternative. Further research should point out whether stimulation with other TLR-agonists and measurement of other cytokines would be of additive value. In the contaminated samples that were used, bacteria were not quantified and identified in all samples. Contamination of the assays had occurred in certain days and confirmed in samples with high background and excluded in samples with lower background. All samples with high background were therefore regarded as contaminated. We assumed that growth \textit{in vitro} induced maximum cytokine levels. Furthermore, as the identified bacteria all have originated from the African soil, their immune response, although varying, might be a best reflection of \textit{in vivo} random stimuli from the environment. A limitation of this study is that we have measured only TNF\textalpha and IL10, which restricts us from making general conclusions on the whole cytokine assay as such. However, based on this data we can speculate about the reproducibility of the pro- and anti-inflammatory cytokine production spectrum.

In this study, comparison between variations in whole-blood stimulation assay induced cytokines from a field-study and a study performed in a well-equipped laboratory are made for the first time. Here we have shown that also in field-based laboratories the whole blood stimulation assay can be used to measure cytokine production. TNF\textalpha production fluctuates more than IL10 production. Therefore TNF\textalpha production from field-studies seems
more to be influenced by environmental stimulations, whereas IL10 better reflects innate pro-or anti-inflammatory tendency. Different TLR-agonists result in different cytokine profiles. As in real-life many stimuli are playing a role in immune regulation, various TLR-agonists should also be represented in a whole-blood stimulation assay. A combination of at least TLR2 and TLR4 ligands seems appropriate to give a broad view of a general innate immune response.

**ACKNOWLEDGMENTS**

This research was supported by the Netherlands Foundation for the advancements of Tropical Research (Grant No. WOTRO 93-467), the Netherlands Organization for Scientific Research (NWO 051-14-050), the EU funded Network of Excellence LifeSpan (FP6 036894) and the Stichting Dioraphte. We want to thank all people who were part of the research team. Furthermore we want to thank Margo van Schie-Troost and Marja Kersbergen-van Oostrom for their work on the cytokine assays.

**REFERENCES**

7. Westendorp RGJ, Langermans JA, Huizinga TW, Elouali AH, Verweij CL, Boomsma DI,
Chapter 3


