Toll-like receptor 4 (TLR4) is the principal receptor for bacterial endotoxin recognition, and functional variants in the gene confer endotoxin hyporesponsiveness in humans (1). Recognition of endotoxin leads to the activation of intracellular signaling pathways, up-regulating a wide array of inflammatory modulators, which contribute to early host-cell response (2, 3).

Recently, a common polymorphism in TLR4 (4) that is associated with hyporesponsiveness to inhaled endotoxin in humans was identified (5–7). This missense mutation (Asp299Gly) in the fourth exon of the TLR4 gene alters the extracellular domain of this receptor. An additional missense polymorphism (Thr399Ile) in the extracellular domain of the TLR4 receptor co-segregates with the Asp299Gly substitution in more than 95% of the Caucasian population (8).

Recent studies showed that genetic variants of TLR4 may contribute to differences in risk in vivo (9) and may modulate in vitro response to bacterial lipopolysaccharide (LPS) (8) related to inflammatory markers. On the basis of these studies, we hypothesized that genetic variants of TLR4 might also contribute to the large interindividual differences in inflammatory marker concentrations after LPS challenge.

The aim of our study was to investigate the association between TLR4 polymorphisms (Asp299Gly and Thr399Ile) and values for inflammatory markers in human experimental endotoxemia.

The study protocols were approved by the Ethics Committee of the Medical University Vienna, and all participants gave written informed consent before entering the study. Complete data and DNA samples were available for 74 healthy male volunteers. These volunteers participated in several clinical trials receiving placebo in addition to endotoxin, including recently published studies (10, 11) and several unpublished trials. All volunteers were 19–35 years of age with a body mass index between the 15th and 85th percentiles.

The LPS model has been described in detail previously (11). All volunteers received 2 ng of LPS (National Reference Endotoxin, Escherichia coli; USP) per kilogram of body weight as a bolus infusion over 2 min.

Blood samples were collected by venipuncture into Vacutainer Tubes containing EDTA as anticoagulant (Becton Dickinson) before LPS infusion and thereafter at the times indicated in Fig. 1. Plasma samples were processed immediately by centrifugation at 2000g at 4 °C for 15 min and stored at −80 °C before analysis. Plasma concentrations of interleukin-6 (IL-6) (11), IL-1β, and tumor necrosis factor (TNF) were measured by a high-sensitivity enzyme immunoassay (R&D-Systems), and all samples from individual participants were run in the same assay.

Plasma concentrations of prothrombin fragment (F1 + 2) and D-dimer were measured by ELISAs from Behring and Roche Diagnostics, respectively (12). Supersensitive C-reactive protein (CRP) values were determined by nephelometry (Tina-quant® CRP; Roche Diagnostics) (11). Blood counts were performed with an XE 2001 cell counter (Sysmex).

Total RNA was prepared with the QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer’s instructions. mRNA was directly transcribed into cDNA by use of the RT-Enzyme Kit (Applied Biosystems) and stored at −80 °C until analysis.

Tissue factor (TF) mRNA was quantified with an ABI Prism 7700 (Applied Biosystems) using primers designed by Primer Express Software (Applied Biosystems) as described previously (10, 13, 14).

Genomic DNA was prepared from frozen whole blood by use of a blood DNA isolation reagent set according to standard procedures (11). Subsequent allele-specific PCR amplification for the TLR4 Asp299Gly and Thr399Ile alleles was performed according to a previously described protocol (15). Genotypes were assigned by independent investigators who were unaware of the participants’ identities.

For statistical analysis, we used the SPSS 10.0 software package (SPSS). The means (SE) are given. The nonparametric Mann–Whitney U-test was used. Because only one individual was homozygous for Asp299Gly and Thr399Ile, he was included in the heterozygous group. A 2-tailed P value <0.05 was considered statistically significant. Correlation of continuous variables was determined by the Spearman correlation coefficient.

Among the 74 persons who received LPS, 63 (85%) were wild type for TLR4 299Asp and 399Thr, 10 (14%) were heterozygous for the less frequent 299Gly allele, and 1 (1%) was homozygous for the TLR4 299Gly allele. In our study population, the Asp299Gly and Thr399Ile polymorphisms were in complete linkage [consistent with the 95% linkage disequilibrium in Caucasians (15)]. Genotype frequencies were in Hardy–Weinberg equilibrium.

Basal and peak values of selected inflammatory markers were not different between wild-type TLR4 individuals and carriers of the 299Gly mutant allele. However, volunteers with the Asp299Gly allele had lower concentrations of some of the inflammatory cytokines, acute-phase reactants, and other mediators of inflammation relatively late after the onset of endotoxemia (Fig. 1 and Table 1).

IL-6 concentrations were lower at 6 h, and CRP and IL-1β concentrations were lower at 24 h after LPS infusion in participants homozygous or homozygous for Asp299Gly (Fig. 1). Additionally, carriers of the Asp299Gly polymorphism had significantly higher monocyte counts 24 h after LPS infusion (Fig. 1).
We also observed trendwise differences in TNF response to LPS after stratification of the data according to TLR4 genotype (data not shown).

Plasma concentrations of F1+2 increased 8-fold 6 h after LPS infusion (P <0.05 vs baseline). Similarly, D-dimer concentrations increased 5-fold with a maximum at 10 h after LPS infusion (Table 1; P <0.05 vs baseline). Maximum TF mRNA transcription occurred at 4 h [mean (SE) 17 (2)-fold] and afterward decreased to basal values (Table 1; P <0.05 vs baseline).

Carriers of the mutant allele had lower F1+2 concentrations at 8 and 24 h after LPS (3 vs 2.1 ng/L at 8 h; 0.8 vs 0.6 ng/L at 24 h; P <0.05), but not D-dimer. This is consistent with the lack of effect on early thrombin generation. In addition, there was no significant association between TF mRNA concentrations and either TLR4 genotype (data not shown).

The observed genotype frequencies of the TLR4 polymorphisms (Asp299Gly and Thr399Ile) were comparable to those reported in other studies (8, 15, 16).

Our data indicate that the TLR4 Asp299Gly polymorphism may influence plasma concentrations of inflammatory mediators in human endotoxemia, particularly at later stages. In mutant 299Gly allele carriers, IL-6 concentrations were significantly lower at 6 h and CRP, IL-1β, and F1+2 concentrations were significantly lower 24 h after LPS infusion (Fig. 1). We found no significant differences, however, between genotypes for basal or peak values of these inflammatory markers. This was a very consistent finding; of note, concentrations of CRP, which is a late inflammation marker and which is determined by IL-6 activation in this model (14), reflect the late differences in IL-6 generation.

As expected (10), monocyte counts decreased sharply after LPS infusion, probably because of margination or trapping in the microcirculation. After 6–8 h, monocytes returned to the bloodstream either from the bone marrow or by demargination. Thus, the increased monocyte counts after 24 h in carriers of the mutant allele could conceivably also reflect an antiinflammatory effect, i.e., demargination of monocytes.

There are conflicting reports on the effects of the Asp299Gly polymorphism on endotoxin responsiveness in vitro (5, 6, 8, 17–19). However, the authors of several clinical reports associated this polymorphism with the risk of gram-negative infections (9, 20) or severe respiratory syncytial viral infection (21) as well as chronic disorders, including airway hyperresponsiveness (8, 22), asthma (23), arteriosclerosis (16), and diabetic neuropathy (24).

The TLR4 299Gly allele has been shown to attenuate human responsiveness to inhaled endotoxin in vivo (22), as well as the increase in leukocyte counts at 6 h and CRP concentrations at 24 h after challenge.

Our study results appear to partially resolve this apparent discrepancy by showing no significant effects on early cytokine release but a decreased inflammatory response in Asp299Gly carriers at a later stage of inflammation (from 6 to 24 h).

The positive study results were obtained mainly in patients with disease duration >24 h (i.e., partly in chronic disease), whereas the negative study results were obtained in short-term in vitro cell cultures (including isolated monocytes). Early cytokine release in our model is dependent on TNF release (25), which in turn is largely driven by the conversion of preformed membrane-bound pro-TNF to active TNF by metalloproteinases (26). Thus, it is conceivable that TLR4 polymorphisms come into play particularly when cytokines must be transcribed, rather than in the very acute phase. However, the exact mechanisms for the predominant contribution of TLR4 polymorphisms in the late rather than very early stages of inflammation are unknown and deserve further investigation.

In summary, our findings indicate that common polymorphisms in TLR4 are associated with differences in the late response to systemic LPS infusion in humans; they also add to current evidence that gene-sequence changes
Table 1. Basal and peak values of inflammatory and coagulation markers in human endotoxemia.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Peak</th>
<th>Influence of Asp299Gly polymorphism on timecourse</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Wild type (n = 63)</td>
<td>Heterozygous (n = 11)</td>
<td></td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>NA(^b)</td>
<td>NA</td>
<td></td>
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<tr>
<td>Time after LPS, 4 h</td>
<td></td>
<td></td>
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<tr>
<td>IL-6, ng/L</td>
<td>1.7 (0.3)</td>
<td>1.3 (0.4)</td>
<td></td>
</tr>
<tr>
<td>Time after LPS, h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TNF, ng/L</td>
<td>54 (11)</td>
<td>46 (20)</td>
<td></td>
</tr>
<tr>
<td>Time after LPS, h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL-1β, ng/L</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Time after LPS, h</td>
<td></td>
<td></td>
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<tr>
<td>TF mRNA, fold increase</td>
<td>1 (0)</td>
<td>1 (0)</td>
<td></td>
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<tr>
<td>Time after LPS, h</td>
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<tr>
<td>F1, mmol/L</td>
<td>0.58 (0.02)</td>
<td>0.57 (0.04)</td>
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<tr>
<td>Time after LPS, h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Dimer, mg/L</td>
<td>0.19 (0.02)</td>
<td>0.12 (0.04)</td>
<td></td>
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<tr>
<td>Time after LPS, h</td>
<td></td>
<td></td>
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</tbody>
</table>

\(^a\) Healthy volunteers (n = 74) received 2 ng/kg LPS, and the influence of the TLR4 Asp299Gly polymorphism on inflammatory response was assessed. Among those who received LPS, 63 were wild type for TLR4 299Asp and 399Thr, and 11 were carriers of the less frequent 299Gly allele. All data are the mean (SE).

\(^b\) Including 1 homozygous individual.

\(^c\) NA, not available.

\(^d\) For peak values.

in TLR4 can alter the ability of the host to respond to environmental stress.

References


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