

# Heterozygous Toll-Like Receptor 4 Polymorphism Does Not Influence Lipopolysaccharide-Induced Cytokine Release in Human Whole Blood

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The heterozygous Asp299Gly mutation of the toll-like receptor (TLR) 4, the key receptor for lipopolysaccharide (LPS), has been associated with attenuated inflammatory responses. When 160 healthy volunteers (9% heterozygous and 0.6% homozygous) were genotyped and their LPS-inducible cytokine release was assessed in an *ex vivo* whole blood test, the responses of heterozygotes did not differ significantly from those of wild-type carriers for any of the cytokines (tumor necrosis factor- $\alpha$ , interleukin [IL]-1 $\beta$ , IL-6, interferon- $\gamma$ , and granulocyte colony-stimulating factor) or eicosanoids measured or for serum cytokines and C-reactive protein. Ten heterozygous subjects and 12 wild-type control subjects responded similarly to a graded series of LPS and *Escherichia coli* concentrations, excluding the possibility that allele-specific differences are evident only at low stimulus concentrations or in response to whole pathogens. These data demonstrate that the heterozygous Asp299Gly polymorphism does not exhibit a functional defect in cytokine release after the stimulation of blood monocytes.

The recognition of bacteria by the immune system and the subsequent induction of inflammatory responses determine the course of infectious diseases. The mechanisms by which pathogens trigger the host's immune response are complex. However, the initial recognition of the pathogens by immune cells represents a crucial step. Only a few years ago, Toll-like receptors (TLRs) were identified as key recognition components of pathogen-associated molecular patterns in mammals, and at least 10 different family members have been identified [1, 2]. TLRs are transmembrane receptors with extracellular leucine-rich repeats and an intracellular signaling domain. Decades ago, C3H/HeJ and C57BL/10ScCr mice were discovered to be incapable of responding to the

bacterial outer surface component lipopolysaccharide (LPS), the main immunostimulatory principle of gram-negative bacteria [3, 4]. In 1998, the *Tlr4* gene was identified by Poltorak et al. [5], and the expression of a nonfunctional TLR4 in C3H/HeJ mice was shown to be the consequence of a point mutation modifying the cytoplasmic domain of TLR4, whereas the loss of LPS responsiveness in C57BL/10ScCr mice was due to a deletion within the gene locus [6]. To date, the important role of TLR4 for the recognition of LPS has been intensely studied by many groups [7–9]. Furthermore, recent work has shown that the TLR4 locus in C57BL/10ScCr mice is haploinsufficient for LPS responses [10].

When the human Asp299Gly polymorphism of TLR4, which cosegregates with the Thr399Ile polymorphism, was described [11], this offered a possible explanation for different susceptibilities toward infection. Indeed, the Asp299Gly, but not the Thr399Ile, mutation was reported to be defective with regard to LPS signaling in transfected THP-1 cells [11]. Furthermore, when 83 healthy subjects were investigated with regard to their airway responsiveness toward in-

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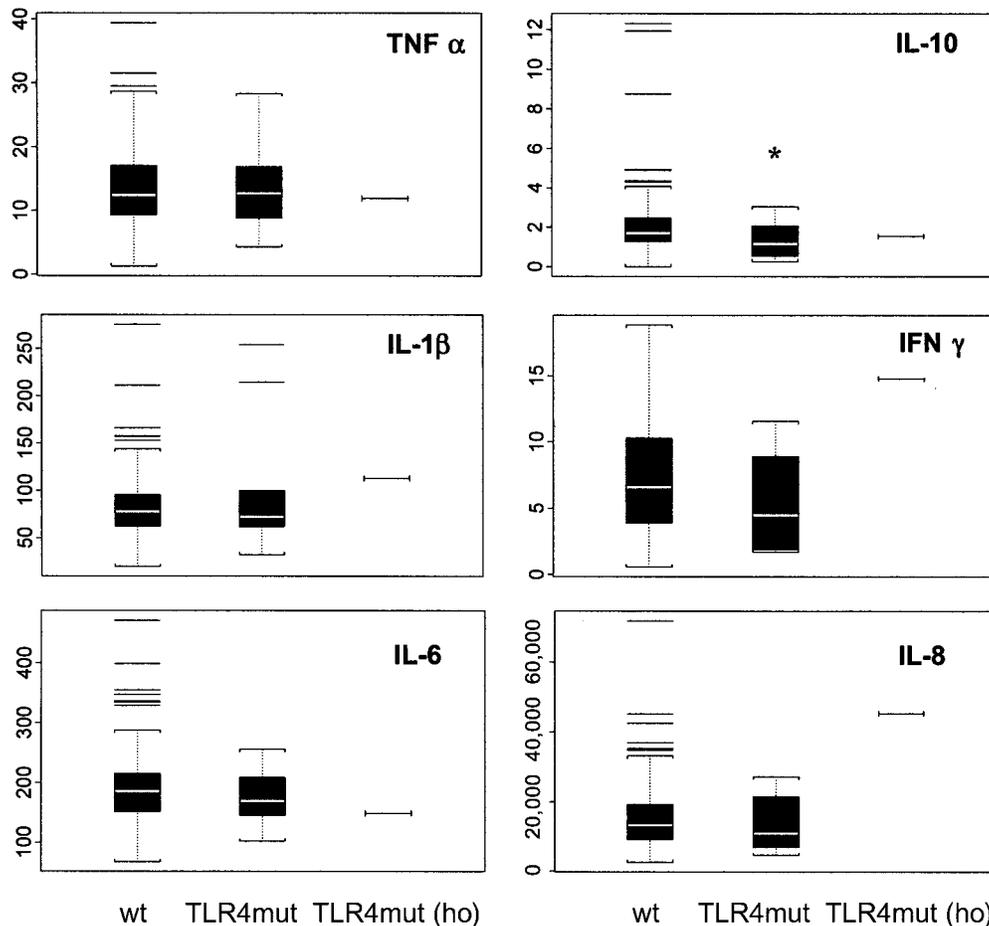
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haled LPS in an incremental challenge test, some of the subjects with the Asp299Gly allele showed reduced forced expiratory volume in response to inhaled LPS. LPS responsiveness was restored in vitro, when airway epithelial cells or alveolar macrophages obtained from these individuals with TLR4 mutation were infected with an adenoviral vector expressing wild-type TLR4 [11, 12]. The results of recent studies have also shown a higher prevalence of gram-negative infections and a more severe course of disease for carriers of the Asp299Gly allele [13, 14]. No association of the Asp299Gly polymorphism with the likelihood of meningococcal disease [15], asthma [16], or *Candida albicans* infections [17] has been found.

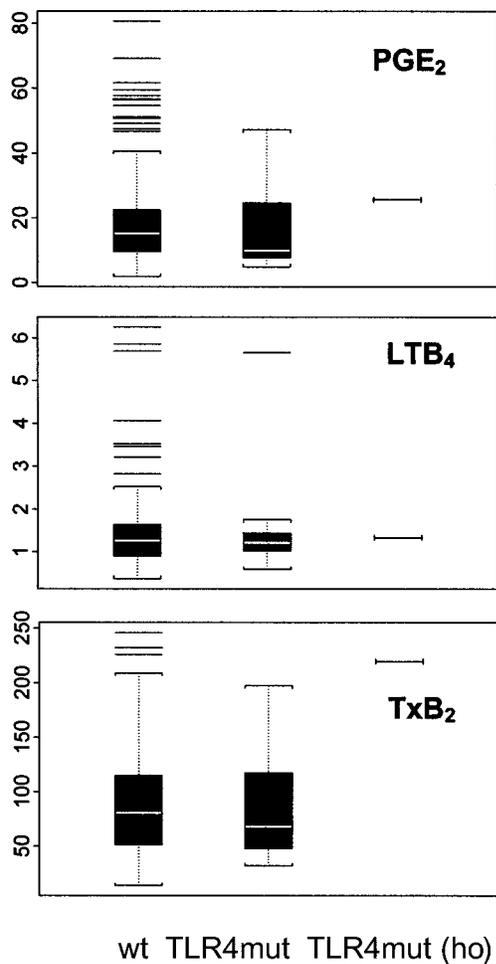
A putative role of infections as triggers of atherosclerosis has been controversial for decades [18–20]. A recent pioneering report demonstrated an attenuated risk of developing atherosclerosis in a cohort of 810 subjects monitored for 5 years when

they were genotyped retrospectively for the Asp299Gly polymorphism [21]. Because not only the progression of carotid atherosclerosis but also inflammatory risk factors such as serum C-reactive protein (CRP) and interleukin (IL)-6 were reduced in heterozygous carriers of this polymorphism [21], these data suggested that differences in the initiation of inflammatory responses might underlie this observation.

The frequency of the Asp299Gly polymorphism of TLR4 ranges from 3% to 11% in the white population. We tested samples from a collective of healthy blood donors ( $n = 160$ ), recruited on 2 consecutive days to minimize interassay variations, with regard to ex vivo inducible inflammatory responses in a standardized human whole-blood incubation [22]. This assay allows the study of monocyte responses to LPS while avoiding preparation artifacts. After genotyping, the data were stratified for heterozygous and homozygous polymorphic do-



**Figure 1.** Influence of Toll-like receptor 4 (TLR4) polymorphism on lipopolysaccharide (LPS)-induced cytokine release. Four milliliters of 20% human whole blood was incubated in the presence of 1  $\mu$ g/mL LPS for 24 h. Cytokines were determined in the cell-free supernatants by ELISA. Cytokine levels are given in nanograms/10<sup>6</sup> monocytes. Data are depicted as a box and whiskers blot that gives the range of values, with the box being subdivided into the 25% and 75% quantiles by the median; 50% of all values lie within the box, and single lines represent outliers. Data are means  $\pm$  SE. Unstimulated controls showed no significant cytokine release. \* $P < .05$ . IFN, interferon; IL, interleukin; TLR4mut(ho), homozygous TLR4 Asp299Gly carrier; TLR4mut, heterozygous TLR4 Asp299Gly carriers; TNF, tumor necrosis factor; wt, wild-type carriers.



**Figure 2.** Influence of Toll-like receptor 4 (TLR4) polymorphism on lipopolysaccharide (LPS)-induced eicosanoid release. Four milliliters of 20% human whole blood was incubated in the presence of 1  $\mu\text{g}/\text{mL}$  LPS for 24 h. Eicosanoids were determined in the cell-free supernatants by EIA. Eicosanoid levels are given in nanograms/ $10^6$  monocytes. Data are depicted as a box and whiskers blot that gives the range of values, with the box being subdivided into the 25% and 75% quantiles by the median; 50% of all values lie within the box, and single lines represent outliers. Data are means  $\pm$  SE.  $\text{LTB}_4$ , leukotrine  $\text{B}_4$ ;  $\text{PGE}_2$ , prostaglandin  $\text{E}_2$ ;  $\text{TLR4mut}(\text{ho})$ , homozygous TLR4 Asp299Gly carrier;  $\text{TLR4mut}$ , heterozygous TLR4 Asp299Gly carriers;  $\text{TxB}_2$ , thromboxane  $\text{B}_2$ ; wt, wild-type carriers.

nors. In addition, the inflammatory serum markers IL-6, tumor necrosis factor (TNF)- $\alpha$ , and CRP were determined.

## MATERIALS AND METHODS

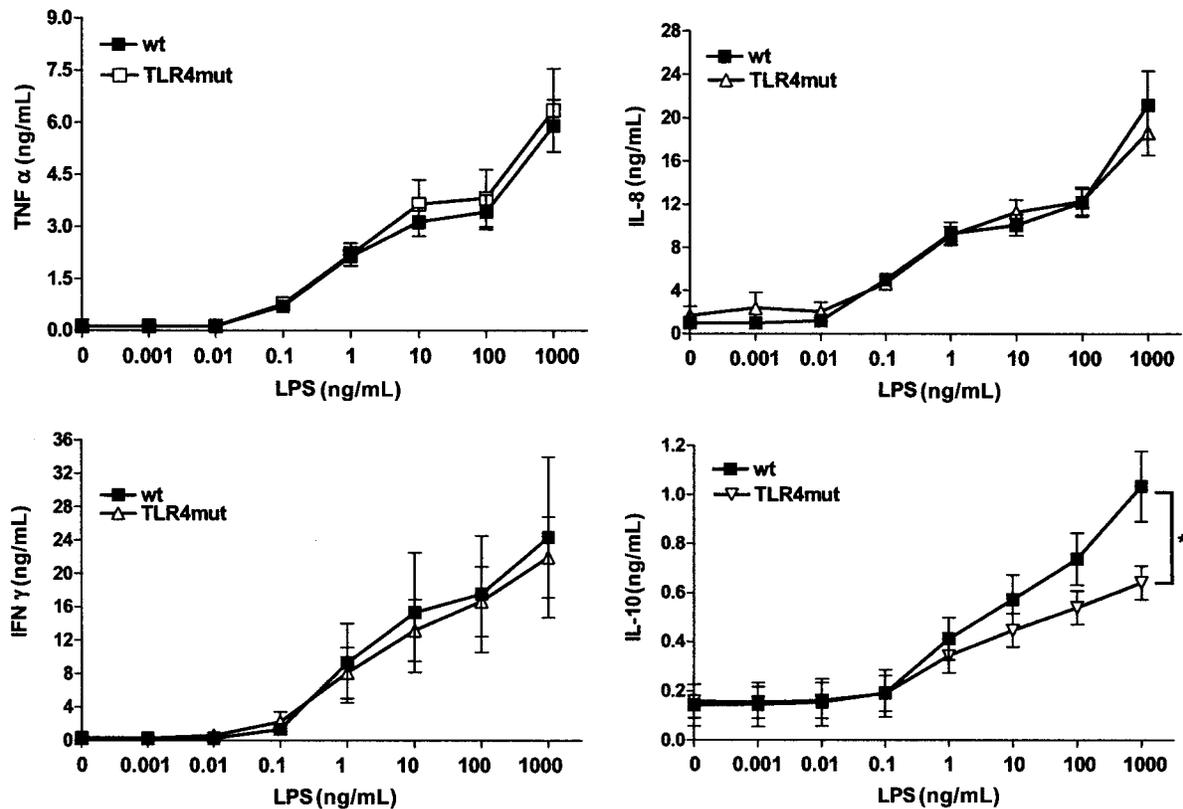
**Volunteer population and TLR4 genotyping.** Serum and blood samples were collected from 160 healthy volunteers recruited at the University of Konstanz (Konstanz, Germany) on 2 consecutive days, within a 2-h period on each day. The mean age of the volunteer population was 30 years (range, 20–70 years), and the sex ratio was 84 men to 76 women. The volunteers were healthy, according to the results of a questionnaire, and had taken

no medication for 1 week prior to blood withdrawal. Differential blood cell counts were measured with a Pentra60 (ABX Technologies) to rule out acute infections. DNA preparation was done using the QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's protocol, including RNase-free DNase digestion. The Asp299Gly TLR4 polymorphism was determined by polymerase chain reaction restriction fragment-length polymorphism [23]. Of these 160 volunteers, 12 subjects with homozygous wild-type alleles and 10 with one Asp299Gly allele were recruited a second time for a blood donation.

**Whole-blood cytokine response.** Incubations of human whole blood in the presence of different stimuli were done as described elsewhere [22]. In brief, heparinized blood freshly taken from healthy volunteers was diluted 5-fold with RPMI 1640 (Bio Whittaker) that contained 2.5 IU of heparin (Liquemin N 25000; Hoffmann LaRoche) and 100 IU penicillin/100  $\mu\text{g}$  streptomycin (Biochrom) per milliliter. As stimuli, LPS from *Salmonella abortus equi* (Sigma), UV-inactivated *Escherichia coli*, the phorbol ester PMA (Sigma), and the exotoxin SEB (Sigma) were used. After the addition of the different stimuli, the samples were incubated in the presence of 5%  $\text{CO}_2$  at 37°C for 24 or 72 h, in case of SEB stimulation. Then, after resuspension, the cells were pelleted by centrifugation (400 g for 2 min), and the cell-free supernatants were stored at  $-70^\circ\text{C}$  for cytokine determination. All experiments and measurements were carried out blindly with regard to the polymorphism status of the donors.

**ELISA.** Cytokines were determined by commercial ELISA for IL-4 (high sensitivity IL-4; Biosource) or with ELISAs based on antibody pairs against human TNF- $\alpha$  and interferon (IFN)- $\gamma$  (Endogen purchased from Perbio Science), IL-5 and IL-10 (BD Biosciences), and IL-2, IL-6, IL-8, and granulocyte colony-stimulating factor (G-CSF; R&D). The binding of biotinylated antibody was quantified using streptavidin-peroxidase (Biosource) and the substrate TMB (3,3',5,5'-tetramethylbenzidine; Sigma). Recombinant cytokines serving as standards were from the National Institute for Biological Standards and Controls (London, United Kingdom; TNF- $\alpha$  and IL-1 $\beta$ ), BD Bioscience (IL-2, IL-4, IL-5, and IL-10), Genzyme (IL-6), Thomae (IFN- $\gamma$ ), Perbio Science (IL-2 and IL-8), and Amgen (G-CSF). Serum TNF was assessed using a high-sensitivity ELISA Quantikine (R&D), and serum CRP was assessed using a highly sensitive CRP assay (Turbimetric test; Roche-Boehringer). Eicosanoid release was determined by commercial ELISAs from Cayman (SPI Europe), according to the manufacturer's instructions, after appropriate dilution.

**Statistics.** Statistical analyses were done using S-Plus 2000 (MathSoft) and GraphPad Prism (version 3.00; GraphPad) software. The *F* test was used to test for equal variances. Data were log-transformed to achieve a Gaussian distribution, followed by *t* test, except for CRP, which was not log transformed



**Figure 3.** Influence of Toll-like receptor 4 (TLR4) polymorphism on lipopolysaccharide (LPS) dose response curves. Two milliliters of 20% human whole blood were incubated in the presence of LPS (1 pg/mL to 1  $\mu$ g/mL) for 24 h. Cytokines were determined in the cell-free supernatants by ELISA. \* $P < .05$ . IFN, interferon; IL, interleukin; TLR4mut, heterozygous TLR4 Asp299Gly carriers ( $n = 10$ ); TNF, tumor necrosis factor; wt, wild-type carriers ( $n = 12$ ).

but was analyzed by the Wilcoxon test. Concentration response curves were subjected to 1-way analysis of variance, followed by Bonferroni's multiple comparison test.

## RESULTS

To examine the association of the TLR4 polymorphism Asp299Gly with levels of basal or inducible proinflammatory cytokines, samples from 160 healthy volunteers were genotyped. Of the 160 subjects, we found 14 (9%) to be heterozygous and 1 (0.6%) to be homozygous for the Asp299Gly polymorphism. When serum levels of the proinflammatory mediators TNF- $\alpha$ , IL-6, and CRP were analyzed and compared between the subjects with the homozygous wild-type and those with the heterozygous Asp299Gly allele, no significant differences were found (table 1). The low CRP levels further continued the absence of ongoing infections or stress. The stimulation of whole blood from all 160 subjects with 1  $\mu$ g/mL LPS also resulted in the release of comparable amounts of the proinflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IFN- $\gamma$ , without any significant difference between the wild-type and polymorphic groups (figure 1). In addition, the LPS-induced release of

G-CSF was not influenced by the polymorphism ( $6 \pm 0.3$  vs.  $5.8 \pm 1.2$  vs.  $5.2$  ng/mL for the wild-type vs. Asp299Gly-heterozygous vs. Asp299Gly-homozygous subjects, respectively). However, the heterozygous polymorphic subjects tended to produce less anti-inflammatory IL-10 ( $P = .002$ ; figure 1). Furthermore, none of the values measured in serum or stimulated blood of the homozygous polymorphism carrier were obviously different from those in the other 2 groups. Because men and women may have different immunological test results, all data were also analyzed by sex, but none of the parameters measured showed sex dependence.

In an extension of this approach, LPS-inducible eicosanoids were determined. Again, no significant differences for LPS-inducible prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), thromboxane B<sub>2</sub>, or leukotriene B<sub>4</sub> were found (figure 2). The same held true for spontaneous cytokine formation in the absence of any stimulus or when non-TLR4 agonists such as the phorbol ester PMA were used to induce the release of TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  or the superantigen SEB to induce the lymphokines IL-2, IL-4, IL-5, and IFN- $\gamma$  (data not shown).

To exclude the possibility that differences in inflammatory responses manifested only at lower levels of stimulation or in

**Table 1. Toll-like receptor 4 (TLR4) polymorphism has no influence on inflammatory serum markers.**

Marker	Wild-type allele (n = 145)			Asp299Gly allele (n = 14)			P <sup>a</sup>
	Median	Mean	SE	Median	Mean	SE	
TNF	3.16	3.36	0.08	3.05	3.17	0.24	.46
Serum IL-6	0.00	115	37	4.40	167	108	.11
CRP	0.071	0.17	0.02	0.075	0.12	0.05	.35

**NOTE.** Data are presented as pg/mL for tumor necrosis factor (TNF), ng/mL for interleukin (IL)-6, and mg/100 mL for C-reactive protein (CRP). The mean values of the homozygous TLR4 carrier ranged within those of the other groups (2.6 pg/mL, 10 ng/mL, and 0.1 mg/100 mL for TNF, IL-6, and CRP, respectively) and are not included in the table.

<sup>a</sup> Wild type vs. Asp299Gly.

response to whole bacteria, 10 of 14 subjects with the Asp299Gly TLR4 polymorphism and 12 randomly chosen wild-type donors from the collective were recruited again, and concentration response curves from 1 pg/mL to 1 µg/mL LPS, as well as incubations with 10<sup>3</sup>, 10<sup>4</sup>, and 10<sup>5</sup> *E. coli*/mL were done, in a blinded fashion. However, as shown in figure 3, both groups responded to increasing concentrations of LPS with similar sensitivity and similar quantities of TNF-α, IL-8, and IFN-γ. Again, the heterozygous TLR4 subjects tended to release less IL-10 than the homozygous wild-type carriers, which became significant at 1 µg/mL (figure 3). The fact that the initial comparison of 14 versus 145 donors already showed such a difference under exactly the same stimulation conditions supports this result, but statistical significance might also be attributed to unbalanced group sizes, in combination with aspects of multiple testing. Incubation with whole bacteria also did not lead to any significant difference in the induction of proinflammatory cytokines (figure 4). Taken together, these data argue against any major functional defect in inflammatory responses in human subjects with the TLR4 polymorphism Asp299Gly.

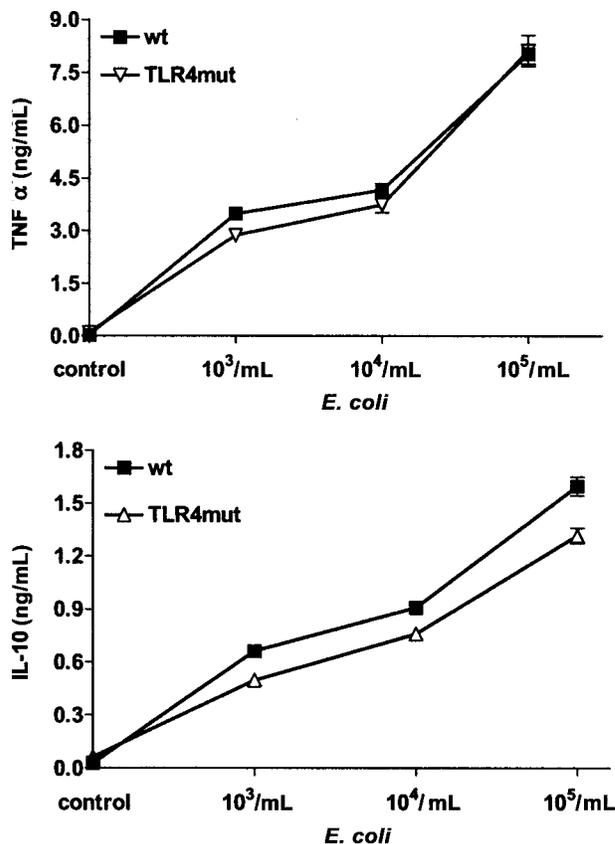
## DISCUSSION

The discovery of dysfunctional polymorphisms in the TLR implied that these might represent risk factors for impaired host response. A higher prevalence of gram-negative infection was reported for carriers of the Asp299Gly allele [13].

We found no difference in the ability of heterozygotes and individuals with 2 wild-type alleles to respond to any gram-negative stimulus. The reduced release of IL-10, which was statistically significant only at the highest LPS concentration, cannot be considered to be biologically relevant. It appears that the defect of the mutated allele is at least partially compensated by the wild-type allele. Also, other LPS-recognizing receptors may play a backup role [24, 25, 26]. This would also explain the sustained response of the single homozygous subject. Unfortunately, he could not be recruited again, but the low in-

cidence of homozygous polymorphism (<1%) makes it a risk factor of lesser demographic importance.

However, the attenuation of bacterial defence due to the TLR4 polymorphism might only become crucial during severe infections. Furthermore, the in vitro LPS stimulation assay can potentially be influenced by the levels of CD14, lipopolysaccharide-binding protein, epinephrine, PGE<sub>2</sub>, and others, which might obscure an impact of the TLR4 mutation. The lack of differences in basal serum levels of TNF-α, IL-6, and CRP demonstrates that there is also no major difference in the basal inflammatory levels of healthy subjects when they are stratified according to polymorphism. These findings also oppose those reported by Kiechl et al. [21], which have also been discussed by others [8, 27], where the reduced risk for atherosclerosis was accompanied by reduced levels of inflammatory mediators. No functional analyses were done in the quoted studies, except for the study of Arbour et al. [11], in which not all hyporesponders in the LPS inhalation test carried the polymorphism and some normal responders were polymorphic, which again



**Figure 4.** Influence of Toll-like receptor 4 (TLR4) polymorphism on cytokine induction by *Escherichia coli*. Two milliliters of 20% human whole blood were incubated in the presence of 10<sup>3</sup>, 10<sup>4</sup>, and 10<sup>5</sup> UV-inactivated *E. coli*/mL for 24 h. Cytokines were determined in the cell-free supernatants by ELISA. IL, interleukin; TLR4mut, heterozygous TLR4 Asp299Gly carriers; TNF, tumor necrosis factor; wt, wild-type carriers

indicates that other factors besides the polymorphism are involved. *E. coli* hardly represents all possible infectious pathogens, and other bacterial or viral pathogens signalling via TLR4 may indeed contribute to the association of TLR4 polymorphism and the progression of atherosclerosis. Heat-shock proteins (hsps) such as human hsp-60 and hsp-70 as well as chlamydial chsp-60 have been shown to require this receptor [28–32]. If endotoxin contamination can really be excluded from those preparations, it would be interesting to test them on the Asp299Gly polymorphism.

To conclude, our data rule out hyporesponsiveness to inflammatory stimulation in heterozygous TLR4 polymorphism carriers, which makes it unlikely that this underlies the reduced risk of TLR4 polymorphic subjects for the progression of atherosclerosis or their increased risk to develop infectious complications. Although ours was essentially a negative study, the lack of any association between the frequent single-nucleotide Asp299Gly polymorphism of TLR4 and the impairment of responses to bacterial stimuli, even in a homozygous individual, contradicts the results of studies that have termed it a risk factor for infectious disease.

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