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Heterozygous Toll-Like Receptor 2 Polymorphism Does Not Affect Lipoteichoic Acid-Induced Chemokine and Inflammatory Responses

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While transfection of *tlr2* conveyed responsiveness to lipoteichoic acid (LTA), the Arg753Gln polymorphic gene could not. LTA induced a stronger chemokine and anti-inflammatory response than lipopolysaccharides did. Blood from heterozygous polymorphic and wild-type donors reacted uniformly to LTA and *Staphylococcus aureus*. Thus, one functional allele for Toll-like receptor 2 suffices for full cytokine response.

Since the discovery of the Toll-like receptors (TLRs) as crucial receptors recognizing microbial components and alerting the immune system (reviewed in reference 4), healthy and patient populations have been screened for polymorphisms in their *tlr2* and *tlr4* genes to determine whether such polymorphisms may be risk factors for bacterial infections. Results obtained up to now have been inconclusive, as only a few carriers of polymorphisms have been identified in small populations and functional assays of the patients' ability to respond to bacterial stimuli were often not performed. We genotyped 160 volunteers for the Asp299Gly polymorphism of *tlr4* and assessed their basal and inducible cytokine release levels. Although the heterozygous Asp299Gly polymorphism has been reported to be a defective polymorphism, our data (10) and a study performed by Erridge et al. (1) do not support any functional consequence of this polymorphism on the inflammatory response.

A mutation screen of 110 healthy volunteers found only one missense mutation of *tlr2* (Arg753Gln) in the open reading frame (5). Functional studies of that polymorphism in transfected cells showed a diminished response to peptides derived from *Borrelia burgdorferi* and *Treponema pallidum*; however, the incidence of the polymorphism was not higher in a septic-shock population than in healthy volunteers (5). The immunostimulatory principles of borreliae and treponemata are not yet clearly defined. We have developed an isolation procedure for lipoteichoic acid (LTA) from gram-positive bacteria which yields highly pure, bioactive LTA (6) that requires TLR2 for signaling (3). LTA from *Staphylococcus aureus* was synthesized chemically on the basis of the proposed structure and found to have biological activity comparable to that of purified natural LTA (8). Thus, we employed LTA from *S. aureus*, *Bacillus subtilis*, and live *S. aureus* to investigate the influence of the Arg753Gln TLR2 polymorphism on inflammatory capacity.

293T cells transfected with wild-type TLR2 as previously described (5) responded to stimulation with LTA from *S. aureus* (6 h at 1.5 μg/ml) with increased NF-κB–luciferase reporter activity (Fig. 1). This significant increase above basal activity was absent in cells transfected with the Arg753Gln polymorphism, indicating that the polymorphism results in a nonfunctional protein.

To be able to compare the cytokine induction potency of LTA from *S. aureus* (prepared in-house as described previously [6, 7], without endotoxin contamination, as indicated by negative *Limulus* amoebocyte lysate assay [QCL-1000; Charles River Endosafe] results) with that of lipopolysaccharides (LPS) in general, we chose three LPS from different bacterial species, i.e., *Klebsiella pneumoniae*, *Escherichia coli* O26:B6, and *Salmonella enterica* serovar Abortusequi (Sigma-Aldrich), which were tested for TLR dependency in C3H/HeJ (TLR4-

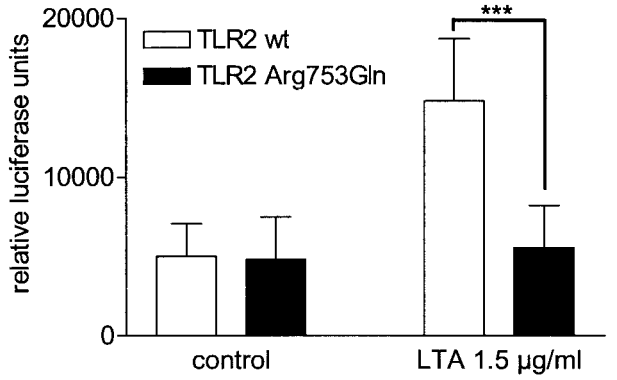

FIG. 1. The Arg753Gln polymorphism of TLR2 is a functional knockout. Shown are the luciferase activities of 293T cells transfected with the wild-type (wt) gene or the Arg753Gln polymorphism for TLR2 and stimulated with 1.5 μg of LTA/ml for 6 h. Averages ± standard deviations for two experiments done in triplicate are shown. ***, $P < 0.001$ (t test).

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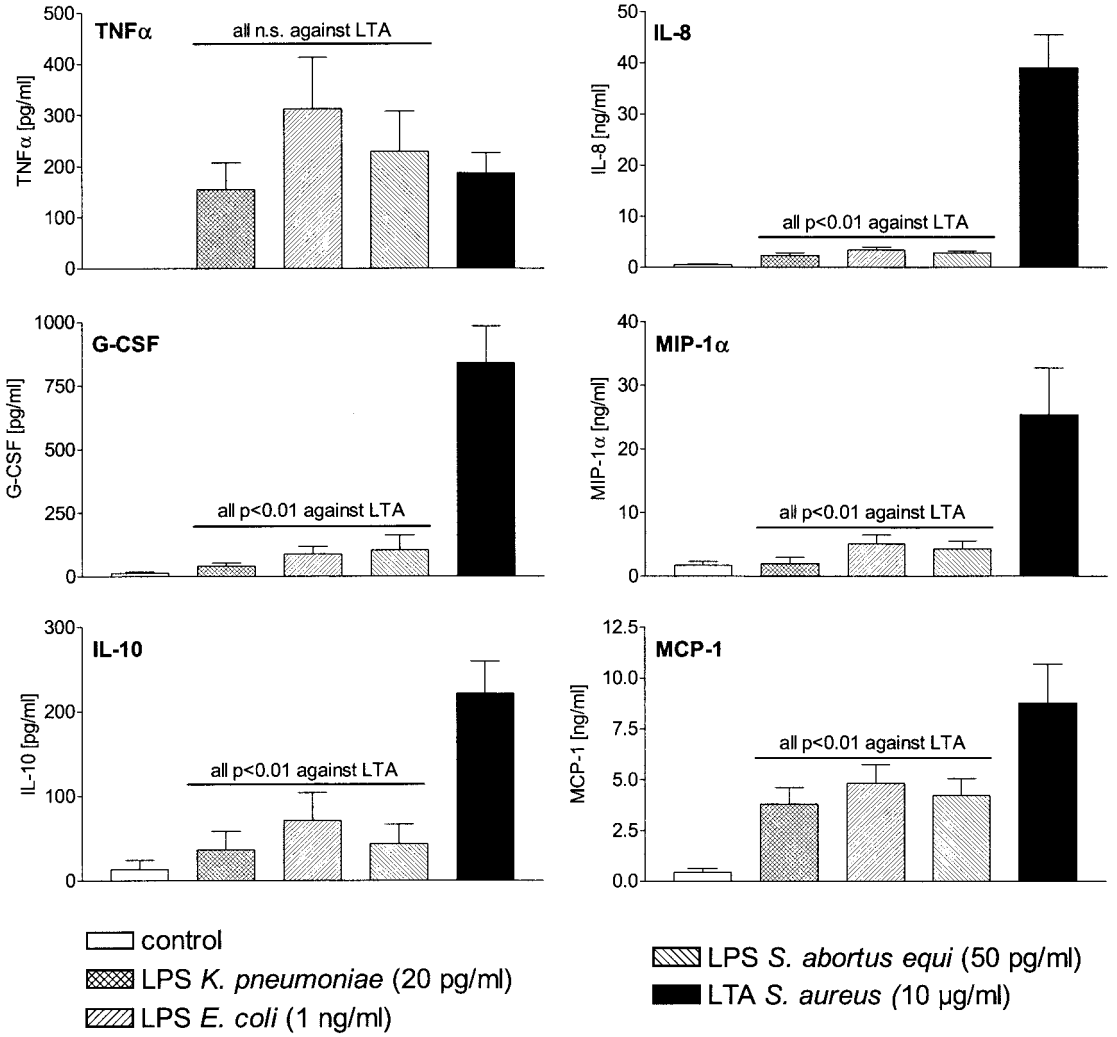

FIG. 2. LTA induces more chemokine and anti-inflammatory activity than four different LPS in concentrations that induce equal amounts of TNF-α. Four LPS from different bacterial species were employed in concentrations inducing the same amount of TNF-α as 10 μg of LTA/ml in a whole-blood incubation. Mediators in the supernatants were measured by ELISA. Data are means ± standard errors of the means for seven healthy donors from one of three similar experiments (repeated-measures analysis of variance followed by Dunnett's multiple-comparisons test). n.s., not significant.

deficient) and TLR2 knockout mice and found to be only TLR4 dependent. These LPS were employed in concentrations that induced the release of the same amounts of tumor necrosis factor alpha (TNF-α) as 10 μg of LTA/ml did, as measured by an enzyme-linked immunosorbent assay (ELISA) after overnight incubation of 20% whole blood performed as previously described (10) (Fig. 2). All LPS, when calibrated to TNF-α release, also induced the release of uniform amounts of the other mediators measured, indicating a common activation pattern of LPS in general. LTA, in comparison to these LPS,

TABLE 1. Cytokine and chemokine induction in whole blood from 12 wild-type and 6 polymorphic donors in response to inflammatory stimuli[a]

Stimulus	TNF-α (pg/ml)		IFN-γ (pg/ml)		IL-8 (ng/ml)		IL-10 (pg/ml)	
	wt	*tlr2* mut	wt	*tlr2* mut	wt	*tlr2* mut	wt	*tlr2* mut
LTA (*S. aureus*) (1 μg/ml)	160 ± 54	144 ± 46	ND	ND	28 ± 9	34 ± 12	209 ± 101	122 ± 32
LTA (*S. aureus*) (10 μg/ml)	321 ± 44	252 ± 89	ND	ND	81 ± 11	85 ± 18	332 ± 98	227 ± 57
Live *S. aureus* (10^4/ml)	58 ± 30	55 ± 23	ND	ND	8.7 ± 3.1	14 ± 7.4	44 ± 26	32 ± 12
Live *S. aureus* (10^5/ml)	1,213 ± 124	1,186 ± 342	1,489 ± 406	842 ± 194	162 ± 55	171 ± 62	240 ± 32	189 ± 58
PMA (100 ng/ml)	4,133 ± 728	5,040 ± 930	6,842 ± 1,199	13,102 ± 2,829	99 ± 15	132 ± 37	157 ± 90	74 ± 22

[a] Data are presented as means ± standard errors of the means (all differences are not significant). wt, homozygous wild-type donors; *tlr2* mut, heterozygous *tlr2* polymorphic donors; ND, not detectable (detection limit, 26 pg/ml).

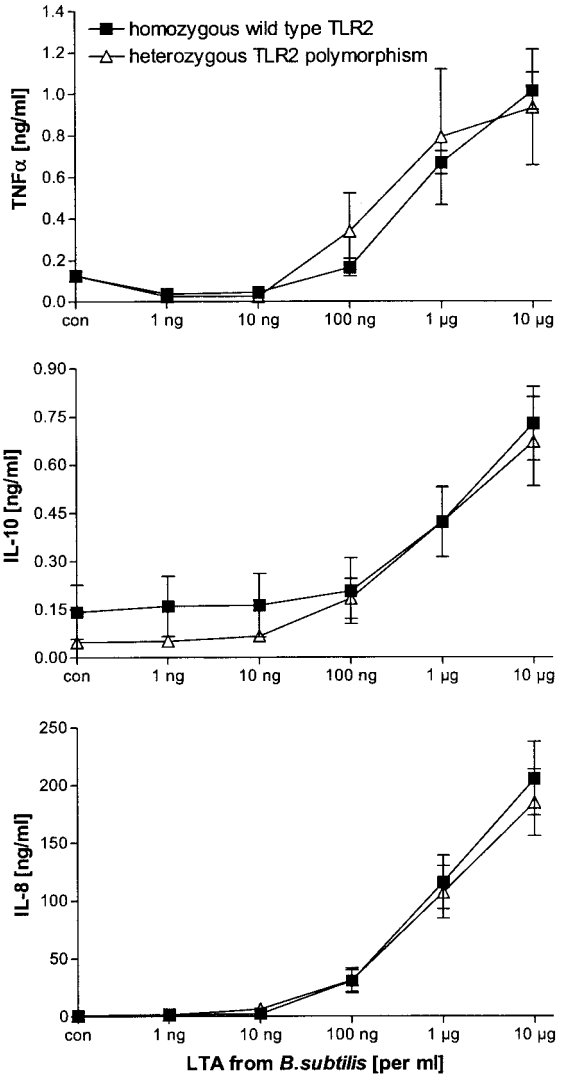

FIG. 3. Effect of heterozygous Arg753Gln TLR2 polymorphism on the cytokine response to LTA from *B. subtilis*. Whole blood from volunteers (12 homozygous wild-type and 6 heterozygous TLR2 individuals) was stimulated with LTA from *B. subtilis*, and cytokine release was measured by ELISA. Data are presented as means ± standard errors of the means (differences were not significant at any concentration).

did not induce measurable gamma interferon (IFN-γ) (data not shown), as expected on the basis of an earlier study (2), but induced significantly higher levels of the anti-inflammatory cytokines granulocyte colony-stimulating factor (G-CSF) and interleukin-10 (IL-10) and of the chemokines (Fig. 2). There are not yet any clear indications in the literature of how the signal transduction pathways differ between LPS and LTA apart from the use of different TLRs, i.e., TLR4 for LPS and TLR2 for LTA (3). The data discussed here strongly indicate that the pathways do not simply converge, and these data demand further investigation. It is tempting to correlate the pronounced induction of IL-8 and G-CSF, both involved in neutrophil attraction and recruitment, by LTA with the well-known pus formation typical for many gram-positive infections. From these data, the cytokines TNF-α, IFN-γ, IL-8, and IL-10 were chosen to represent the overall cytokine pattern induced by the two different stimuli.

To investigate the impact of the Arg753Gln TLR2 polymorphism on inflammatory responses, we screened DNA of 160 healthy volunteers by restriction fragment length analysis as previously described (9) and found a heterozygous TLR2 polymorphism prevalence of 6.3%. Blood from 6 heterozygous subjects and 12 wild-type controls was stimulated in parallel (by experimenters who were blind to the blood's TLR2 status) with LTA from *B. subtilis* and *S. aureus*, live *S. aureus* (DSM 20233), or receptor-independent phorbol ester (PMA), and the levels of the proinflammatory cytokines TNF-α and IFN-γ, the chemokine IL-8, and the anti-inflammatory cytokine IL-10 released were measured. As shown in Fig. 3, the subjects with the heterozygous polymorphism responded to LTA from *B. subtilis* with sensitivities and quantities of cytokine release similar to those of the wild-type subjects. Furthermore, the cytokine responses of the two groups to LTA from *S. aureus*, live *S. aureus*, or PMA were not significantly different for any parameter measured (Table 1). The batch of LTA from *S. aureus* employed in these experiments was less potent in inducing cytokine responses than the *B. subtilis* preparation. However, the lower potency of *S. aureus* LTA represents no general phenomenon.

Taking these data together, we report for the first time responses to highly purified LTA which are stronger than those inducible by LPS titrated to equal TNF-α induction levels, showing that LTA is not just a weaker endotoxin but also a potent stimulus in its own right. The transfection results presented here show that the TLR2 polymorphism at position 753 is a functional knockout of LTA stimulation, similar to the TLR4 polymorphism at position 299 for LPS. However, the inflammatory response of heterozygous carriers of the polymorphism to TLR2 agonists was not different from that of wild-type controls, indicating that one allele carrying a nonfunctional TLR2 polymorphism is compensated by the second allele. These data complement previous studies on the TLR4 polymorphism, which found no difference in the responses to LPS in the blood of heterozygous donors and even one homozygous donor (1, 10). Polymorphisms in the TLRs do not appear to be responsible for differences in cytokine responses or, by extrapolation, vulnerability to bacterial infections.

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