The Functional Variant (Asp299gly) of Toll-like Receptor 4 (TLR4) Influences TLR4-Mediated Cytokine Production in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To investigate functional consequences of the Toll-like receptor 4 (TLR4) variant (Asp299Gly) in rheumatoid arthritis (RA).

Methods. Peripheral blood mononuclear cells from 28 patients with RA carrying or not carrying the TLR4 variant were incubated with lipopolysaccharide (LPS) and heat shock protein B8 (HSPB8). Concentrations of interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), and IL-10 were determined along with TLR4 and CD14 expression.

Results. TLR4 expression was similar in patients carrying or not carrying the variant. In contrast, both LPS and HSPB8 resulted in significantly lower secretion of IL-6, TNF-α, and IL-10 in those who carried the variant, whereas the frequency of CD14+ cells was higher in these individuals.

Conclusion. TLR4 variant clearly reduces its potency to mediate signaling. Correction for CD14+ cells is necessary in comparable experiments. (First Release Mar 15 2008; J Rheumatol 2008; 35:558–61)

Key Indexing Terms:
TOLL-LIKE RECEPTORS RHEUMATOID ARTHRITIS DENDRITIC CELLS

The innate immune system provides an essential mechanism against microbial pathogens. Toll-like receptors (TLR) comprise a family of pattern-recognition receptors that form the first line of defense against invading microbes. Recent research shows that TLR are also implicated in the recognition of "self" molecules[1,2]. These so-called endogenous ligands are released upon tissue damage, which is likely to occur in rheumatoid arthritis (RA). Accumulating evidence points to a role of TLR in RA. TLR are highly expressed in RA synovium, and cells from patients with RA produce higher amounts of inflammatory mediators upon TLR-mediated activation[3,4]. Recently, the involvement of TLR in arthritis was substantiated using various experimental models of arthritis[5-7].

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Arbour, *et al* described a common missense mutation (Asp299Gly) affecting TLR4 function, rendering individuals hyporesponsive to lipopolysaccharide (LPS). In addition, transfection of THP-1 cells with the TLR4 variant revealed a decreased response to LPS[8]. Other studies confirmed the relation between this variant and disease phenotypes[9,10]. Yet similar research in RA is controversial[11-13]. Following the observations by Arbour, *et al*, several groups studied the functional consequences of this variant. However, due to contrasting results these studies have left the issue unresolved. Since TLR4 is potentially involved in the pathogenesis of RA, we investigated whether the TLR4 variant has functional consequences for TLR expression and TLR-mediated cytokine production in RA. We observed that cytokine production by CD14+ cells carrying the TLR4 variant was significantly lower compared to their counterparts not carrying the variant, although the expression of TLR4 was unaffected.

MATERIALS AND METHODS

Study population. A total of 14 patients with RA heterozygous for the Asp299Gly TLR4 variant (AG) and 14 patients without this variant (AA) were studied. Patients fulfilled the American College of Rheumatology criteria for RA and gave informed consent. Patients under treatment with so-called "biologicals" [anti-tumor necrosis factor-α (TNF-α) preparations] and high-dose prednisolone were excluded from the study.

Genotyping and real-time polymerase chain reaction (PCR). Genotyping of the TLR4 Asp299Gly polymorphism (869 A→G; rs4986790) was performed as described[12]. Measurement of TLR4 expression by quantitative real-time PCR was performed using the ABI-Prism 7000 sequence detection system. The following primers were used: hGAPDH forward 5'-ATC TTC TTT TGC GTC GCC AG-3' and reverse 5'-TTC CCC ATG GTG TCT

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GAG C-3'; TLR4 forward 5'-GGC ATG CCT GTG CTG AGT T-3' and reverse 5'-CTG CTA CAA CAG ATA CTA CAA GCA CAC T-3'. Quantification of PCR signals was performed by comparing the cycle threshold value (C_t) with the C_t values of the reference gene GAPDH (ΔCt).

Generation and culture of peripheral blood mononuclear cells (PBMC), characterization, and cytokine measurement. PBMC were isolated using density gradient centrifugation over Ficoll-Paque. Cells were washed and subsequently cultured in a concentration of 0.5×10^6 cells/ml in RPMI-1640 Dutch Modification, supplemented with 10% fetal calf serum for 16 h. 200 ng/ml LPS or 10 µg/ml heat shock protein B8 (HSPB8) and endogenous TLR4 agonist was added. Antibodies used for flow cytometry were anti-human CD14, CD3, TLR4, and the appropriate (isotype) control antibodies. We used the standard protocol for flow cytometry. After 24 h, supernatants were collected. TNF-α, IL-6, and IL-10 levels were measured using the Bio-Plex system®.

Statistical analysis. Differences between groups were analyzed by Mann-Whitney U-test or chi-square where appropriate. The level of significance was $p < 0.05$.

RESULTS

Patient characterization and TLR4 genotype. A total of 14 RA patients with the Asp299Gly TLR4 variant (AG) and 14 RA patients without the variant (AA) were studied. No significant differences in demographic and clinical disease characteristics were observed (Table 1).

Table 1. Demographic and clinical characteristics of RA patients with (A/G) and without (A/A) genetic TLR4 variant.

Characteristic	A/G, n = 14	A/A, n = 14	p
Age, yrs	66 (SD 10)	63 (SD 11)	NS
% Women	55	62	NS
Mean DAS28 score	3.3 (SD 1.3)	3.2 (SD 1.3)	NS
% RF-positive	78	87	NS
Age at onset, yrs	54.3 (SD 8.7)	55.7 (SD 12.3)	NS
Disease duration, yrs	11.8 (SD 6.3)	10.0 (SD 5.6)	NS
% patients using DMARD*	89	78	NS

*No RA patient was using more than 1 DMARD at the same time. DMARD: disease modifying antirheumatic drug, DAS28: Disease Activity Score, NS: nonsignificant.

TLR variant did not express TLR4. We determined levels of TLR4 mRNA expression in freshly isolated PBMC. Real-time PCR analysis showed that levels of TLR4 mRNA expression were not statistically different between RA patients' cells with and those without the Asp299Gly variant

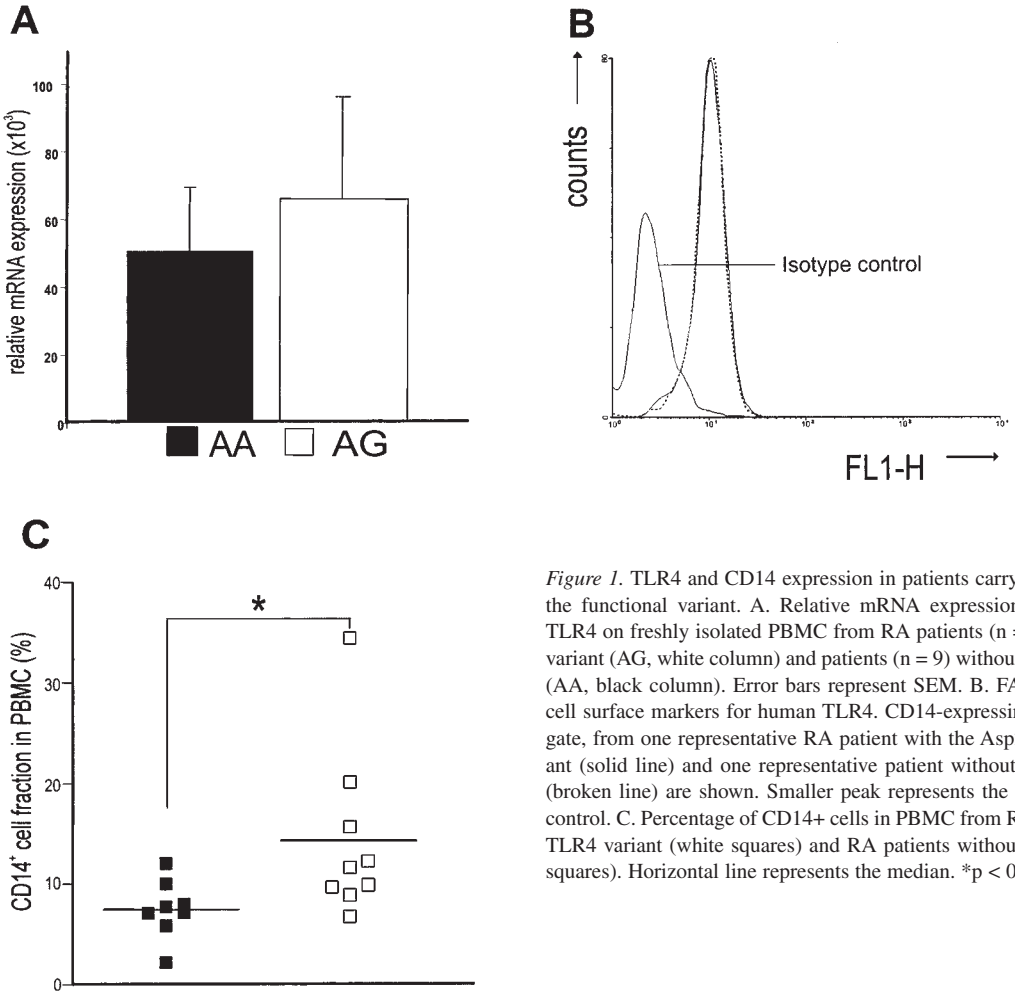

Figure 1. TLR4 and CD14 expression in patients carrying or not carrying the functional variant. A. Relative mRNA expression ($2^{-\delta Ct}$) of human TLR4 on freshly isolated PBMC from RA patients (n = 9) with the TLR4 variant (AG, white column) and patients (n = 9) without the polymorphism (AA, black column). Error bars represent SEM. B. FACS analysis of the cell surface markers for human TLR4. CD14-expressing cells, within life gate, from one representative RA patient with the Asp299Gly TLR4 variant (solid line) and one representative patient without the polymorphism (broken line) are shown. Smaller peak represents the appropriate isotype control. C. Percentage of CD14+ cells in PBMC from RA patients with the TLR4 variant (white squares) and RA patients without the variant (black squares). Horizontal line represents the median. *$p < 0.05$.

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(Figure 1A). Even 24 h after TLR stimulation, the expression of TLR4 was similar between the 2 groups, excluding a secondary effect on TLR expression of the stimuli used (data not shown). In accord with the PCR results, FACS analysis showed that TLR4 expression on the CD14+ cell fraction was similarly distributed among cells from RA patients with and without the TLR4 variant (Figure 1B). TLR4 expression was not detectable on CD3+ cells.

Since CD14 is an adaptor molecule for TLR4 signaling, we next studied CD14+ expression in the PBMC fraction to exclude potential biases in the functional assays. Unexpectedly, the percentage of CD14+ cells was higher (mean ± SEM 14.4% ± 2.8%) in patients carrying the variant compared to those who did not (mean ± SEM 7.5% ± 1.0%; Figure 1C).

Decreased TLR-mediated cytokine production in patients carrying the TLR4 variant. To further investigate the functional consequences of the TLR4 variant we stimulated PBMC from RA patients with and without the variant with an exogenous (LPS) and an endogenous (HSPB8) TLR4 agonist. After correcting for the number of CD14-positive cells, LPS induced significantly lower levels of IL-6 (61%), TNF-α (66%), and IL-10 (56%) in patients carrying the variant. The same trend was observed for HSPB8, in which a comparable decreased production of IL-6 (54%), TNF-α (69%), and IL-10 (41%) was observed, indicating that the TLR variant is truly functional (Figure 2).

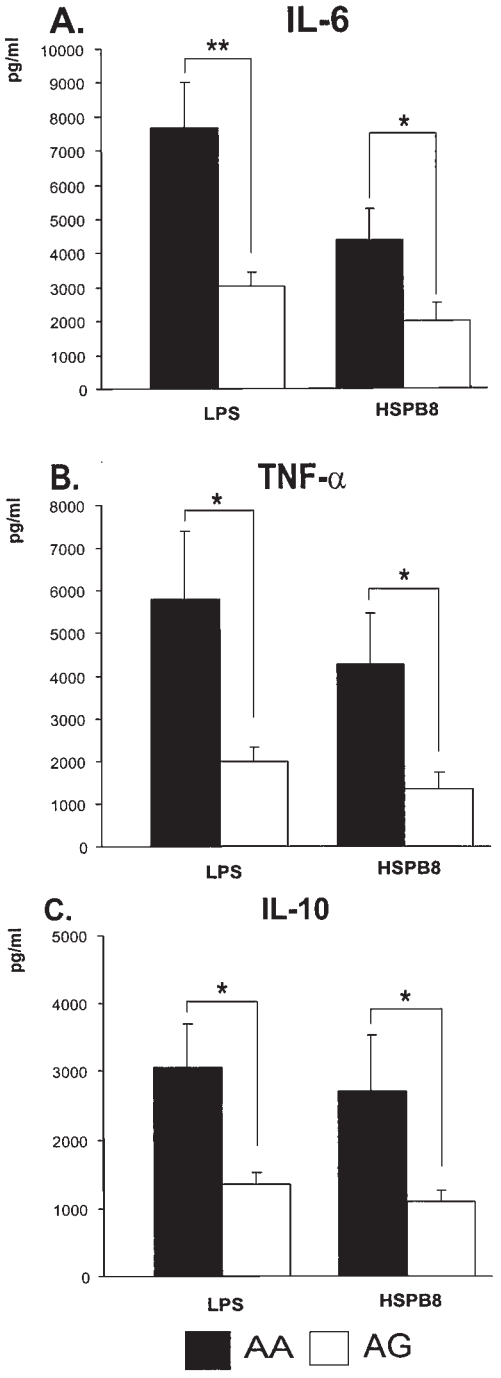

Figure 2. Secretion of inflammatory mediators [IL-6 (A), TNF-α (B), IL-10 (C)] is markedly decreased upon TLR4-mediated activation. Mean cytokine production by PBMC from RA patients (n = 14) with the TLR4 variant (AG) and RA patients (n = 14) without the TLR4 variant (AA) after stimulation with LPS (200 ng/ml) and HSPB8 (10 μg/ml) for 24 h. Error bars represent the SEM. The sensitivity of the cytokine assay was < 5 pg/ml for each measured cytokine. *p < 0.05; **p < 0.01.

DISCUSSION

We demonstrated that the Asp299Gly TLR4 polymorphism had no influence on the levels of expression of mRNA and protein of TLR4, but in contrast, cell stimulation via TLR4 led to a clearly decreased secretion of inflammatory mediators. Several groups have recently investigated the functional consequences of the Asp299Gly TLR4 polymorphism. Schippers, *et al*[14] and Rittersma, *et al*[15] found that the TLR4 variant had no functional consequences in terms of cytokine release in whole-blood samples from patients undergoing cardiovascular surgery. In contrast, but in agreement with the observations by Arbour, *et al*[8], epithelial cells from subjects heterozygous for the Asp299Gly TLR4 polymorphism were found to be functionally hyporesponsive[11]. Interestingly, all studies dealing with this subject that concluded that the Asp299Gly TLR4 polymorphism did not lead to impaired cytokine responses did not correct for the number of CD14-positive cells, including that by van der Graaf, *et al*[17]. Sabroe, *et al* demonstrated that neutrophils, which are abundantly present in whole blood, are scarcely involved in TLR4-induced cytokine production, although they do express low levels of TLR4[18]. These neutrophils are likely to be activated by inflammatory mediators from TLR4-stimulated monocytes, leading to production of proinflammatory cytokines that is independent from TLR4. Consistent with our data, it is conceivable that cytokine production by indirectly activated neutrophils or other cell types present in whole blood veil the effects of the Asp299Gly polymorphism on TLR4-bearing mononuclear

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cells, casting doubt on the conclusions drawn from previous studies. Although we show that the TLR4 variant in patients with RA results in a diminished TLR4-mediated response, these patients have significantly higher quantities of CD14+ cells. This may explain why RA patients do not show remarkable effects of the polymorphism in terms of disease severity or susceptibility[12], although the Asp299Gly TLR4 polymorphism is associated with an impaired immune response.

We observed that the TLR4 variant has a clear influence on TLR4-mediated cytokine secretion.

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