

TOLL-LIKE RECEPTOR 4 POLYMORPHISMS AND ATHEROGENESIS

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ABSTRACT

Background The ability to mount a prominent inflammatory response to bacterial pathogens confers an advantage in innate immune defense but may signal an increased risk of atherosclerosis. We determined whether recently discovered genetic variants of toll-like receptor 4 (TLR4) that confer differences in the inflammatory response elicited by bacterial lipopolysaccharide are related to the development of atherosclerosis.

Methods As part of the five-year follow-up in the Bruneck (Italy) Study, we screened 810 persons in the study cohort for the TLR4 polymorphisms Asp299Gly and Thr399Ile. The extent and progression of carotid atherosclerosis were assessed by high-resolution duplex ultrasonography.

Results As compared with subjects with wild-type TLR4, the 55 subjects with the Asp299Gly TLR4 allele had lower levels of certain proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules, such as interleukin-6 and fibrinogen. Although these subjects were found to be more susceptible to severe bacterial infections, they had a lower risk of carotid atherosclerosis (odds ratio, 0.54; 95 percent confidence interval, 0.32 to 0.98; $P=0.05$) and a smaller intima-media thickness in the common carotid artery (regression coefficient, -0.07 ; 95 percent confidence interval, -0.12 to -0.02 ; $P=0.01$).

Conclusions The Asp299Gly TLR4 polymorphism, which attenuates receptor signaling and diminishes the inflammatory response to gram-negative pathogens, is associated with a decreased risk of atherosclerosis. This finding is consistent with the hypothesis that innate immunity may play a part in atherogenesis. (N Engl J Med 2002;347:185-92.)

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INDUCTION of inflammation is an important component in the defense against microorganisms. Owing to the putative proatherogenic effects of intravascular inflammation, we hypothesized that an efficient innate immune defense may offer an early advantage at the expense of chronic vascular damage in later years.

The responsiveness of the individual innate immune system has a complex genetic background and depends on the virulence of given pathogens. Recently, a family of receptors — the toll receptors — has been described that provides a critical link between immune stimulants produced by microorganisms and the ini-

tiation of host defense.¹⁻⁴ Activation of these receptors results in the release of antimicrobial peptides, inflammatory cytokines, and costimulatory molecules that initiate adaptive immunity (Fig. 1).² For infections with gram-negative bacteria, lipopolysaccharide is the main source of inflammation, and toll-like receptor 4 (TLR4) is crucial in mediating its effects.¹⁻⁴ TLR4 is expressed on cardiomyocytes, macrophages, airway epithelia, and endothelial and smooth-muscle cells and in small amounts in most other tissues.^{2,5} Although the relation between TLR4 and lipopolysaccharide has been the most thoroughly investigated, TLR4 also interacts with other exogenous and endogenous ligands, including respiratory syncytial virus, heat-shock proteins, fibronectin, fibrinogen, and hyaluronic acid (Fig. 1).^{2,6-9}

The recent characterization of the human TLR4 polymorphisms Asp299Gly and Thr399Ile, which impair the efficacy of lipopolysaccharide signaling and the capacity to elicit inflammation,¹⁰ offers a good opportunity for a critical evaluation of the hypothesis that innate immunity plays a part in atherogenesis. For that purpose, we assessed the two TLR4 variants in a random sample of the general population and analyzed the relations among these polymorphisms, the level of systemic inflammation, the risk of severe infections, and the development of atherosclerosis.

METHODS**Study Subjects**

The Bruneck Study is a prospective population-based survey of the epidemiology and pathogenesis of atherosclerosis.¹¹⁻¹³ The study protocol was reviewed and approved by the appropriate ethics committees, and all study subjects gave their written informed consent. At the 1990 base-line evaluation, the study population was recruited as a random sample, stratified according to sex and age, of all inhabitants of Bruneck, Italy (125 women and 125 men in each of the following age groups: 40 to 49 years, 50 to 59 years, 60 to 69 years, and 70 to 79 years). A total of 93.6 percent of those recruited participated, and data assessment was completed for 919 subjects. Between 1990 and the reevaluation in the summer of 1995 (the first five-year period) 63 subjects died or

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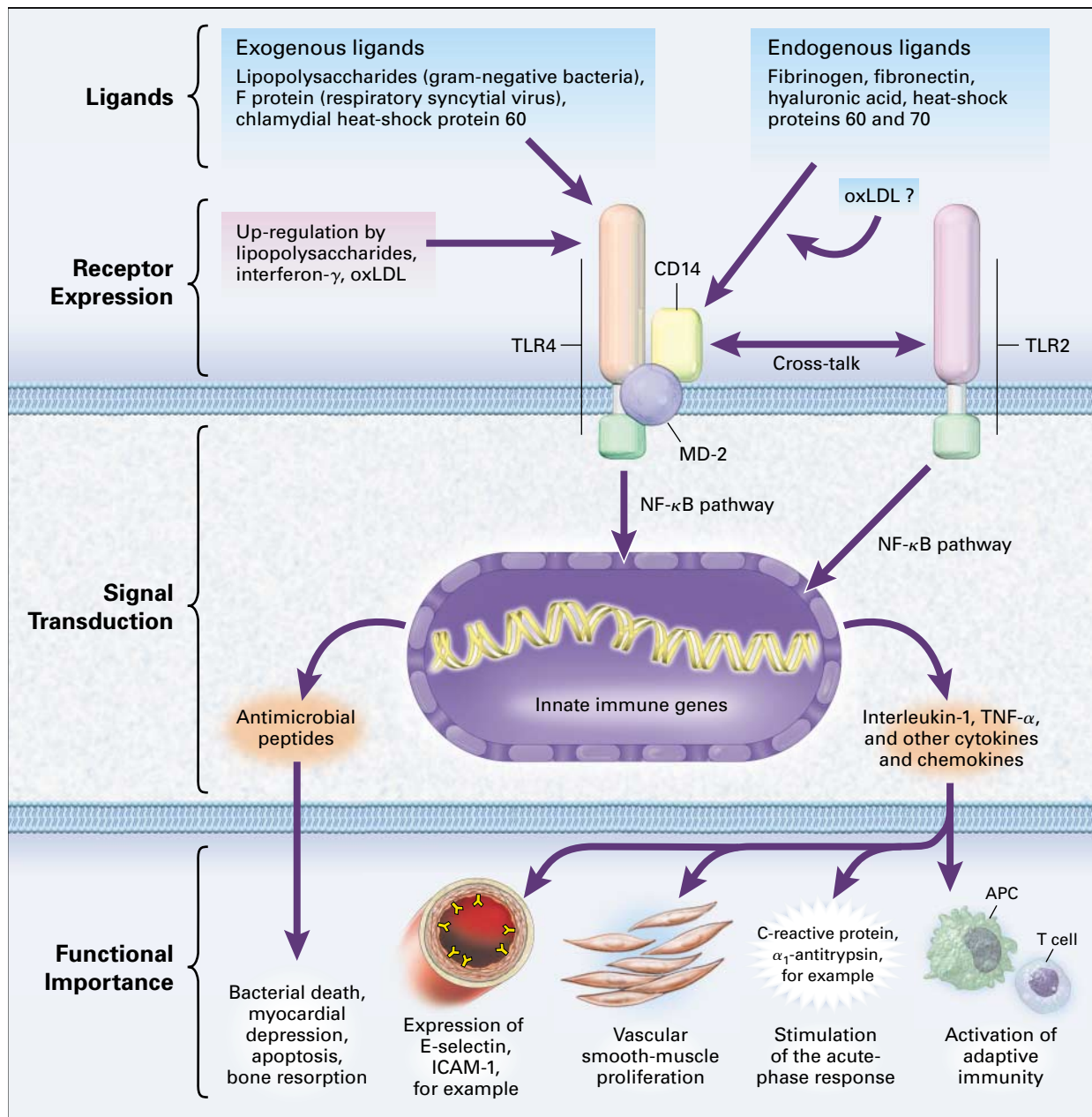


Figure 1. Toll-like Receptor 4 (TLR4).

Shown are candidate ligands, the means of regulation of receptor expression, mechanisms of signal transduction, and the functional importance in various tissues. It is likely but not yet certain that oxidized low-density lipoprotein (oxLDL) has a role as a ligand of TLR4. TLR2 denotes toll-like receptor 2, NF- κ B nuclear factor- κ B, TNF- α tumor necrosis factor α , APC antigen-presenting cell, and ICAM-1 intracellular adhesion molecule 1.

moved away. Among survivors who remained in Bruneck, follow-up was 96.5 percent complete (826 subjects had complete data for the first period). Blood specimens for DNA extraction were drawn as part of the follow-up in 1995. No adequate polymerase-chain-reaction (PCR) products were obtainable from 16 samples, so 810 men and women were included in the main analysis. Of

these subjects, 94 died between the summer of 1995 and the summer of 2000 (the second five-year period). During the second period, 100 percent of the subjects (810) were followed up for newly diagnosed cardiovascular disease, and 94.3 percent of the 716 survivors (675 subjects) underwent sonographic reevaluation.

Clinical History Taking and Examination

Hypertension was defined as a blood pressure (mean of three measurements) of 160/95 mm Hg or higher or the use of anti-hypertensive drugs. Subjects were considered to have diabetes mellitus if they had a fasting glucose level of at least 140 mg per deciliter (7.7 mmol per liter), a glucose level two hours after oral glucose challenge of at least 200 mg per deciliter (11.1 mmol per liter), or both.

Information on severe acute infections of bacterial origin including pneumonia, pyelonephritis, peritonitis, diverticulitis, and sepsis was collected during the second five-year follow-up period from a detailed self-reported medical history, medical records provided by general practitioners, death certificates, and reviews of the data bases of the Bruneck Hospital, which is the only hospital in the district. Chronic infections were assessed by an extensive screening procedure, as detailed previously.¹⁴

Assessment of newly diagnosed (fatal and nonfatal) cardiovascular disease during the second period was based on the patient's medical history, a detailed review of the data bases of the Bruneck Hospital and of death certificates, and the results of clinical and various laboratory examinations.¹⁵ Myocardial infarction was considered confirmed when the World Health Organization criteria for definite disease status were met.¹⁶ Stroke and transient ischemic attack were classified according to the criteria of the National Survey of Stroke.¹⁷ The diagnosis of peripheral artery disease required a positive response to the Rose questionnaire, with the vascular nature of problems confirmed by standard diagnostic procedures.

Laboratory Methods

Blood samples were drawn after an overnight fast and 12 hours of abstinence from smoking.¹⁸ In subjects with acute infection, the drawing of blood was delayed for at least six weeks — that is, until at least four to five weeks after recovery from the infectious illness. Markers of infection and inflammation were measured with commercial assays as follows: C-reactive protein, α_1 -antitrypsin, and ceruloplasmin by nephelometry (Behring); soluble vascular-cell adhesion molecule 1, soluble intracellular adhesion molecule 1, and E-selectin by enzyme-linked immunosorbent assay (R&D Systems and Bender); soluble interleukin-2 receptor by enzyme immunoassay (T Cell Diagnostics); neopterin by radioimmunoassay (Henning), and interleukin-6 by enzyme amplified sensitivity immunoassay (Biosource).

DNA Extraction and TLR4 Genotyping

Genomic DNA was prepared from frozen whole blood with the use of a blood DNA isolation kit (Genomic Prep, Amersham Pharmacia Biotech). Subsequent allele-specific PCR amplification for the TLR4 alleles Asp299Gly and Thr399Ile was performed according to a previously described protocol.¹⁹ Genotypes were assigned by independent investigators who were unaware of the patients' identities and phenotypes.

Assessment of Atherosclerosis

The protocol for ultrasonography involved the scanning of the internal carotid arteries (bulbus and distal segments) and the common (proximal and distal segments) carotid arteries on both sides with a 10-MHz imaging probe.¹⁸ Atherosclerotic lesions were defined according to two ultrasonographic criteria: the wall surface (the presence of protrusions or roughness on the arterial boundary) and the wall texture (whether or not it was echogenic). The maximal axial diameter of plaques was assessed in each of the eight vessel segments, and an atherosclerosis score was calculated by summing all diameters (intraobserver coefficient of variation in a random subgroup ["reproducibility sample"] consisting of 100 persons in the Bruneck population, 13.5 percent).¹⁸ In addition,

the development of new carotid plaques (new cases of atherosclerosis) was assessed in all subjects (kappa coefficients in the reproducibility sample, >0.8).^{11,12} The intima-media thickness was quantified at the far wall of plaque-free sections of the common carotid arteries as the distance between the interface of the lumen and the intima and the interface of the media and the adventitia (intraobserver coefficient of variation in the reproducibility sample, 7.9 percent).¹²

Statistical Analysis

Analysis of variance was used to compare levels of circulating mediators of inflammation in subjects with wild-type TLR4, those with the Asp299Gly allele, and those with both the Asp299Gly allele and the Thr399Ile allele. In supplementary analyses, the levels in subjects with wild-type TLR4 were compared with those in the other two groups combined. The association of TLR4 polymorphisms with newly diagnosed carotid atherosclerosis was examined by logistic-regression analysis with a test procedure determined by the maximum-likelihood estimator.²⁰ A base model was adjusted only for age, sex, and presence or absence of atherosclerosis at base line. Multiple regression analyses were adjusted for a fixed set of covariates that were assessed in previous analyses of the vascular risk profiles of the Bruneck study population.¹³ Genetic categories were modeled as sets of indicator variables. The presence of differential effects of TLR4 variants on the risk of atherosclerosis in various subgroups was tested by the inclusion of interaction terms. Logistic-regression models were supplemented and confirmed by linear regression analyses in which the log-transformed atherosclerosis score or the intima-media thickness was used as a continuous outcome variable. Finally, crude and adjusted hazard ratios for newly diagnosed cardiovascular disease were calculated by Cox models.²¹ All P values are two-sided.

RESULTS

Of the 810 men and women tested, 53 were heterozygous for the Asp299Gly TLR4 allele, and 2 were homozygous, for an allelic frequency of 3.1 percent and a carriage rate of 6.0 percent (with adjustment for the age and sex distribution of the general population in Bruneck). In 46 of these subjects (25 men and 21 women), cosegregation of the Thr399Ile polymorphism was observed, whereas 9 subjects (7 men and 2 women) had an isolated Asp299Gly polymorphism.

As compared with the carriers of the wild-type TLR4, subjects with the Asp299Gly allele (alone or in combination with the Thr399Ile allele) had lower levels of some of the inflammatory cytokines, acute-phase reactants, soluble adhesion molecules, and other mediators of inflammation that we tested (Table 1). In contrast, no associations were observed between TLR4 genotypes and common vascular risk factors or lifestyle factors.

Carriers of TLR4 polymorphisms appeared to be more susceptible to bacterial infections. In the current study population, 53 subjects (6.5 percent) had severe acute infections of putative bacterial origin during the second five-year period. The frequency of such infectious illness was substantially higher among subjects with the Asp299Gly allele but not the Thr399Ile allele (3 of 9 [33 percent]) and subjects with both

polymorphisms (5 of 46 [11 percent]) than among those with wild-type TLR4 (45 of 755 [6 percent]; $P=0.002$ by the chi-square test). In contrast, chronic infections occurred at similar rates in the various groups (4 of 9 [44 percent], 16 of 46 [35 percent], and 242 of 755 [32 percent], respectively; $P=0.69$ by the chi-square test).

Next, we estimated the potential effects of TLR4

polymorphisms on carotid-artery disease with the use of three distinct ultrasonographic measures of the severity and progression of atherosclerosis: intima-media thickness in the common carotid artery (measured in 1995) — a frequently used surrogate measure for systemic vessel disease; an atherosclerosis summary score¹⁸ (calculated in 1995); and the person-based model of atherosclerosis progression de-

TABLE 1. ASSOCIATIONS BETWEEN COMMON POLYMORPHISMS OF TOLL-LIKE RECEPTOR 4 AND LEVELS OF MARKERS OF INFLAMMATION IN THE 810 SUBJECTS.*

VARIABLE	WILD-TYPE TLR4 (N=755)	ASP299GLY+, THR399ILE+ (N=46)	ASP299GLY+, THR399ILE- (N=9)	P VALUE†	
				3-WAY COMPARISON	WILD-TYPE VS. COMBINED POLYMORPHISM GROUPS
Interleukin-6 (pg/ml)	8.6±18.5	5.9±3.6	3.9±1.8	0.04	0.04
Adjusted mean	9.6	6.7	4.7		
Procalcitonin (pg/ml)	29.5±21.6	24.1±10.6	26.1±9.5	0.05	0.03
Adjusted mean	31.9	26.8	20.3		
C-reactive protein (mg/liter)	3.72±7.80	2.65±4.10	1.40±1.94	0.09	0.08
Adjusted mean	3.49	2.46	1.16		
α ₁ -Antitrypsin (mg/dl)	199±37	195±31	192±38	0.22	0.11
Adjusted mean	202	201	191		
Ceruloplasmin (mg/dl)	27.0±5.3	25.8±3.8	24.5±4.2	0.15	0.08
Adjusted mean	26.9	26.4	24.6		
Fibrinogen (mg/dl)	290±76	271±46	239±38	0.01	0.003
Adjusted mean	297	263	238		
Ferritin (ng/ml)	149±168	126±102	117±60	0.08	0.03
Adjusted mean	170	143	84		
Soluble ICAM-1 (ng/ml)	329±95	312±56	302±49	0.10	0.07
Adjusted mean	329	314	299		
Soluble VCAM-1 (ng/ml)	680±242	557±76	535±98	0.03	0.02
Adjusted mean	726	584	526		
E-selectin (ng/ml)	54.1±21.3	49.0±15.0	49.4±14.0	0.22	0.20
Adjusted mean	54.1	50.6	47.5		
Soluble interleukin-2 receptor (U/ml)	316±292	236±152	250±97	0.11	0.15
Adjusted mean	330	251	250		
Neopterin (nmol/liter)	7.9±2.7	7.4±2.6	6.2±1.2	0.01	0.004
Adjusted mean	8.3	7.6	6.1		

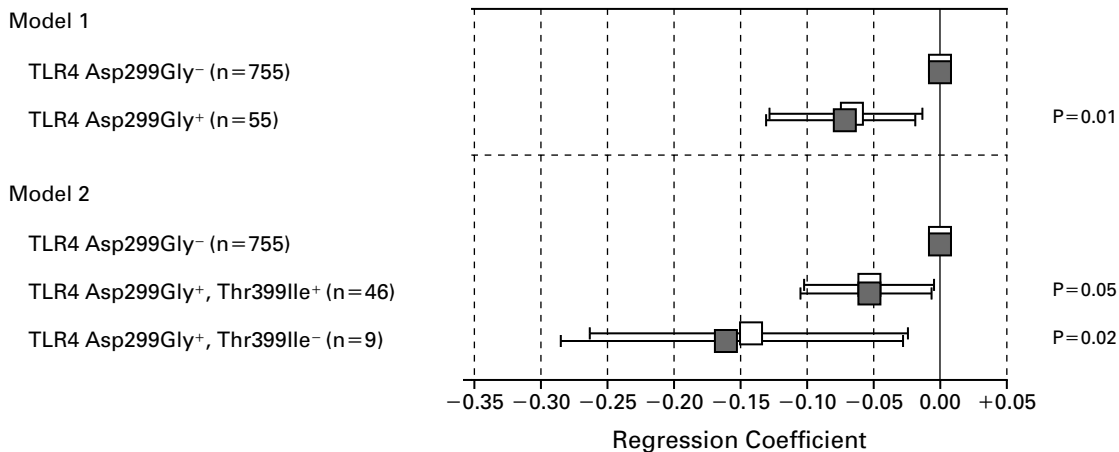
*Plus-minus values are unadjusted means ±SD. Adjusted means were adjusted for age and sex.

†P values were derived from analysis of variance (covariates, age and sex). In the group with wild-type TLR4, the mean age was 58 years and the proportion of men was 50 percent; in the group with both Asp299Gly and Thr399Ile alleles, the mean age was 59 years and the proportion of men was 54 percent; and in the group with Asp299Gly but not Thr399Ile, the mean age was 67 years and the proportion of men was 78 percent. The abbreviation ICAM-1 denotes intracellular adhesion molecule 1, and VCAM-1 vascular cell adhesion molecule 1.

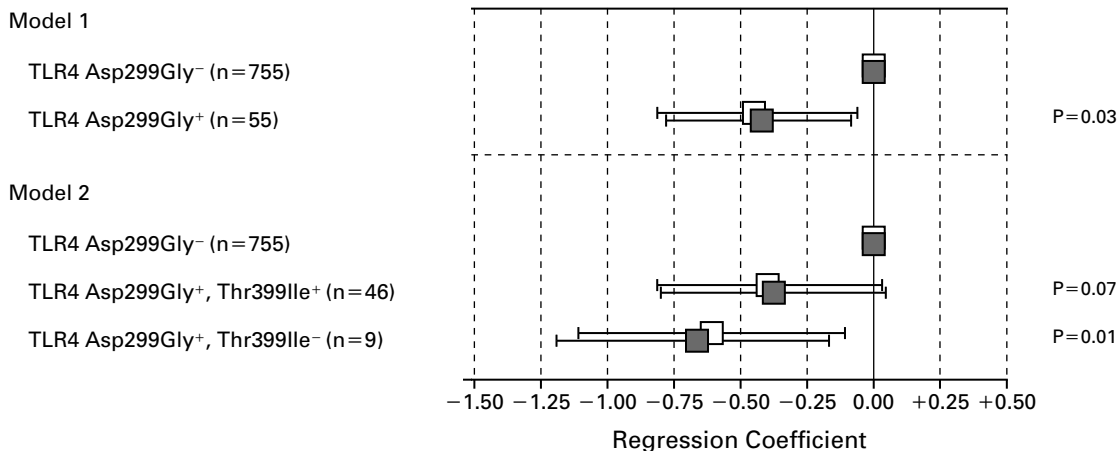
Figure 2 (facing page). Associations between Common Toll-like Receptor 4 Polymorphisms and Measures of the Severity and Progression of Atherosclerosis.

Odds ratios and regression coefficients were adjusted for age and sex (open squares) and for levels of low-density and high-density lipoprotein cholesterol and of lipoprotein(a), presence or absence of hypertension, smoking status, level of alcohol consumption, ferritin level, presence or absence of diabetes, and presence or absence of microalbuminuria (solid squares). The P values given were derived from the multiple regression analyses. Separate models were fitted for the comparison of subjects without the Asp299Gly allele (Asp299Gly⁻) and the combined group of subjects with the Asp299Gly allele (Asp299Gly⁺) (model 1) and for the comparisons of subjects without the Asp299Gly allele, those with both the Asp299Gly allele and the Thr399Ile allele (Asp299Gly⁺, Thr399Ile⁺), and those with Asp299Gly only (Asp299Gly⁺, Thr399Ile⁻) (model 2). Horizontal bars represent the 95 percent confidence intervals.

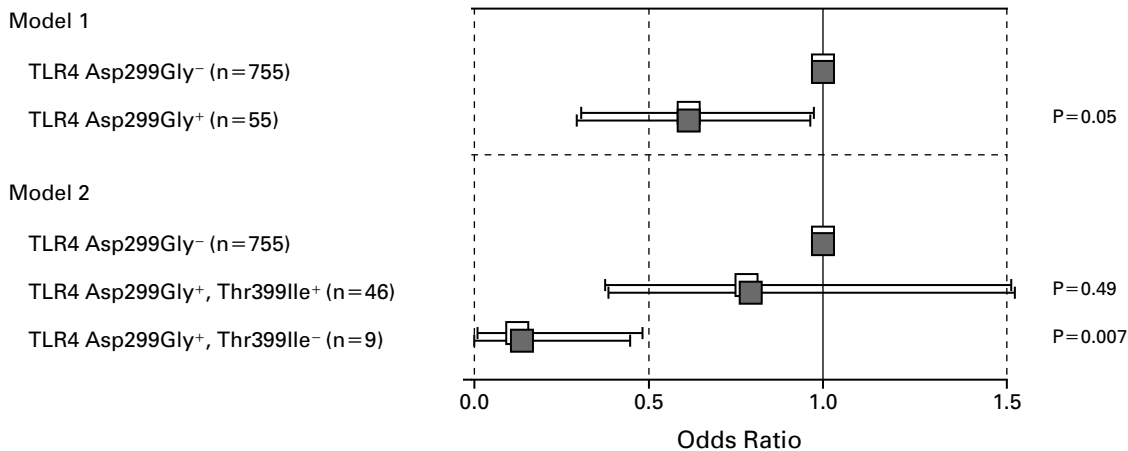
Common carotid-artery intima-media thickness (1995)



Log-transformed carotid atherosclerosis score (1995)



Newly diagnosed carotid atherosclerosis (first period)



veloped specifically for the Bruneck Study (applied during the first five-year period).^{11,12} Results of these analyses are summarized in Figure 2. Whatever measure of atherosclerosis was applied, the Asp299Gly TLR4 allele emerged as a significant protective factor. Beneficial effects appeared to be more pronounced in the 9 subjects with the isolated Asp299Gly polymorphism than in the 46 subjects with both Asp299Gly and Thr399Ile. The age- and sex-adjusted mean intima-media thickness was 1006 μm in subjects with wild-type TLR4 and 937 μm in those with the Asp299Gly allele (difference, 69 μm). Corresponding values for the atherosclerosis score were 4.26 mm and 3.03 mm (difference, 1.23 mm).

All of the above results were virtually unchanged when the statistical models were adjusted for common vascular risk factors (Fig. 2). There was no evidence of differential effects of TLR4 polymorphisms in subgroups defined according to the level of risk or lifestyle factors.

To demonstrate the consistency of our findings over a longer period, the computations were repeated with data from the second five-year follow-up period. These analyses yielded associations between TLR4 polymorphisms and atherosclerosis similar to those found in the original evaluation. Despite the fact that fewer study subjects (675) were involved, most relations found were statistically significant. The regression coefficients for the comparison between subjects with the Asp299Gly allele and those with wild-type TLR4 were as follows: intima-media thickness (measured in 2000), -0.046 (95 percent confidence interval, -0.090 to -0.001 ; $P=0.04$) with adjustment for age and sex, and -0.042 (95 percent confidence interval, -0.085 to 0.001 ; $P=0.06$) according to the multivariate model; five-year changes in the intima-media thickness (during the second follow-up period), -0.050 (95 percent confidence interval, -0.099 to -0.001 ; $P=0.05$), with adjustment for age and sex, and -0.053 (95 percent confidence interval, -0.102 to -0.004 ; $P=0.03$) according to the multivariate model. The odds ratio for newly diagnosed atherosclerosis (during the second follow-up period) was 0.47 (95 percent confidence interval, 0.22 to 1.03; $P=0.06$) with adjustment for age and sex; according to the multivariate analysis, the odds ratio was 0.43 (95 percent confidence interval, 0.24 to 0.94; $P=0.04$).

Subjects with the Asp299Gly allele were less likely to have cardiovascular disease during the second follow-up period: the age- and sex-adjusted hazard ratio was 0.16 (95 percent confidence interval, 0.02 to 1.24; $P=0.08$). Similar proportions of the subjects who died during the second period (8 of 94 [8.5 percent]) and of those who survived (47 of 716 [6.6 percent]) had the Asp299Gly polymorphism; how-

ever, none of the 8 deaths in subjects with the polymorphism had cardiovascular causes, whereas 28 of the 86 deaths in subjects with wild-type TLR4 had cardiovascular causes ($P=0.10$ by Fisher's exact test).

DISCUSSION

TLR4 is the transmembrane lipopolysaccharide receptor that initiates the innate immune response to common gram-negative bacteria, including *Chlamydia pneumoniae* and *Helicobacter pylori* — the two pathogens most commonly implicated in human atherogenesis.^{1-4,22,23} Lipopolysaccharide binding activates the transcription factor nuclear factor- κB , ultimately leading to the synthesis and release of antimicrobial peptides, inflammatory cytokines and chemokines, and costimulatory molecules that provide a critical link to adaptive immunity (Fig. 1).¹⁻⁴ These inflammatory mediators can exert various atherogenic effects involving the expression of adhesion molecules on endothelial cells, proliferation of smooth-muscle cells, activation of immune cells, and stimulation of the acute-phase response (Fig. 1). Recently, numerous potential activators of TLR4 signaling and expression distinct from lipopolysaccharide have been suggested (Fig. 1).^{2,6-9,24,25}

In this investigation, we demonstrated that the presence of the common Asp299Gly polymorphism of TLR4, which affects the composition and structure of the extracellular domain of the receptor, predicted low levels of circulating inflammatory molecules and conferred an increased risk of severe infections but a reduced risk of atherosclerosis. The same polymorphism has been shown to attenuate human responsiveness to inhaled endotoxin in vivo and to interrupt TLR4-mediated lipopolysaccharide signaling in cellular-transfection studies and in airway epithelial cells from heterozygous persons; the latter effect may be corrected by the addition of wild-type TLR4.¹⁰

Our findings have several implications. First, part of the low-grade systemic inflammation that is measurable in healthy subjects appears to be mediated by the TLR4 pathway. Circulating bacterial lipopolysaccharide, a potent ligand of TLR4, is detectable in virtually all healthy persons, albeit at highly variable concentrations.²⁶ Toxic lipoteichoic acid from gram-positive bacteria, mycobacterial lipids, and proteins from respiratory syncytial virus are also recognized by TLR4, and it has been proposed that there is functional "cross-talk" among the members of the TLR family.²⁷⁻²⁹ Therefore, the innate inflammatory defense against a broad palette of microorganisms may rely on an intact TLR4 pathway, and the immune system is commonly exposed to many of these pathogens. Recent findings indicate that the importance of TLR4 may well extend beyond antimicrobial defense to other inflammatory processes inherently related to human

atherogenesis (Fig. 1).^{4,8,9,25} Human heat-shock protein 60 has been shown to require functional TLR4 in order to stimulate the production of tumor necrosis factor α and nitric oxide.⁸ It has been proposed that heat-shock protein 60 acts as a “danger antigen” that, because of stimulation by various endogenous and exogenous stress factors, is overexpressed and partly released into the circulation, where it exerts inflammatory effects, thus driving the innate defense system to increased alertness.^{2,8} Moreover, oxidized low-density lipoprotein up-regulates TLR4 messenger RNA expression in vitro, and the expression of TLR4 by macrophages is preferentially increased in lipid-rich plaques in humans.²⁵ In aggregate, these findings indicate that TLR4 is stimulated by multiple agents that bridge inflammation, infection, and hyperlipidemia.

Second, diminished levels of intravascular inflammation and the enhanced risk of severe bacterial infections observed in subjects with the Asp299Gly TLR4 polymorphism are clear indications of an attenuated innate immune defense. We have recently found that this allelic variant of TLR4 predisposes persons to septic shock with gram-negative microorganisms.³⁰

Third, our study documents that a genetic variant rendering persons susceptible to acute disseminated bacterial infection confers a decreased risk of atherosclerosis. Conversely, carriers of the wild-type allele, who are therefore capable of producing a prominent inflammatory response to virulent gram-negative pathogens, appear to have an increased risk of atherosclerosis. There are two sources of indirect support for our findings. On the one hand, the level of systemic inflammation, irrespective of its origin, has been shown to be a prominent predictor of atherosclerosis in humans,³¹⁻³³ and it has been conjectured that high levels of circulating endotoxin have similar predictive value.^{26,34} On the other hand, proinflammatory polymorphisms of other (key) components of the inflammatory cascade, such as polymorphisms of interleukin-6, E-selectin, tumor necrosis factor α , and CD14, have been linked to an increased risk of cardiovascular disease.³⁵⁻³⁸ It should, however, be noted that our epidemiologic finding of a lower risk of atherosclerosis among carriers of TLR4 polymorphisms lacks confirmation in animal studies. In recent experiments by Wright and colleagues, there was no difference in the extent of aortic atherosclerosis between standard apolipoprotein-E-deficient mice and mice that were also deficient in TLR4 or mice raised in a germ-free environment.³⁹ In this mouse model of atherogenesis, TLR4 appeared to be unrelated to the emergence of vessel disease, at least in the absence of standardized exposure to pathogens.

In interpreting our data, several limitations and

potential sources of bias should be considered. Individual measurements of markers of inflammation may not necessarily reflect the long-term activation of the inflammatory cascade. In the current study, however, some of the markers of inflammation were measured twice or three times at five-year intervals, and the associations with genetic TLR4 variants emerged consistently. We cannot exclude the possibility that TLR4 variants serve as markers of another important genetic abnormality without themselves being functionally relevant; none of the gene products encoded close to the TLR4 region on chromosome 9 have yet been found to be related to atherosclerosis. Finally, a differential loss to follow-up could have influenced the finding of a reduced risk of atherosclerosis among subjects with the Asp299Gly allele. Such a bias, however, appears unlikely, given the nearly complete follow-up, the small number of Asp299Gly-positive subjects among those who died, and the underrepresentation of deaths from cardiovascular causes in the group with the Asp299Gly allele.

In conclusion, the common Asp299Gly TLR4 polymorphism, which attenuates receptor signaling and diminishes the inflammatory response to gram-negative pathogens and, potentially, to other relevant ligands, is associated with low levels of certain circulating mediators of inflammation and a decreased risk of atherosclerosis. These data provide epidemiologic support for the concept that an efficient innate immune defense against bacteria and associated long-term intravascular inflammatory stress are involved in the development of atherosclerosis.

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