Published in final edited form as:
*Hum Genet*. 2011 June ; 129(6): 655–662. doi:10.1007/s00439-011-0962-4.

# Genetic variants in *TLR2* and *TLR4* are associated with markers of monocyte activation: the Atherosclerosis Risk in Communities MRI Study

**Suzette J. Bielinski**
Division of Epidemiology, Department of Health Sciences Research, Mayo Clinic, Harwick Building 6-56, 200 First Street South West, Rochester, MN 55909, USA

**Jennifer L. Hall**
Department of Medicine Developmental Biology Center, Lillehei Heart Institute, University of Minnesota, Minneapolis, MN, USA

**James S. Pankow**
Division of Epidemiology and Community Health, University of Minnesota, Minneapolis, MN, USA

**Eric Boerwinkle**
Human Genetics Center, University of Texas-Houston Health Science Center, Houston, TX, USA

**Nena Matijevic-Aleksic**
Division of Hematology, University of Texas Health Science Center, San Antonio, TX, USA

**Max He**
Department of Biostatistics, School of Public Health, University of North Carolina, Chapel Hill, NC, USA

**Lloyd Chambless**
Department of Biostatistics, School of Public Health, University of North Carolina, Chapel Hill, NC, USA

**Aaron R. Folsom**
Division of Epidemiology and Community Health, University of Minnesota, Minneapolis, MN, USA

## Abstract

Markers of monocyte activation play a critical role in atherosclerosis, but little is known about the genetic influences on cellular levels. Therefore, we investigated the influence of genetic variants in monocyte differentiation antigen (*CD14*), toll-like receptor-4 (*TLR4*), toll-like receptor-2 (*TLR2*), and myeloperoxidase (*MPO*) on monocyte surface receptor levels. The study sample consisted of 1,817 members of a biracial cohort of adults from the Atherosclerosis Risk in Communities Carotid MRI Study. Monocyte receptors were measured using flow cytometry on fasting whole blood samples. *TLR2* rs1816702 genotype was significantly associated with CD14+/TLR2+ percent of positive cells (%) and median fluorescence intensity (MFI) in whites but not in blacks ($p < 0.001$). Specifically, the presence of the minor T-allele was associated with increased receptor levels. In blacks, *TLR4* rs5030719 was significantly associated with CD14+/TLR4+ monocytes (MFI) with mean ± SE intensities of 16.7 ± 0.05 and 16.0 ± 0.14 for GG and



Correspondence to: Suzette J. Bielinski.
bielinski.suzette@mayo.edu.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00439-011-0962-4) contains supplementary material, which is available to authorized users.

GT/TT genotypes, respectively ($p < 0.001$). Variants in *TLR2* and *TLR4* were associated with monocyte receptor levels of TLR2 and TLR4, respectively, in a biracial cohort of adults. To our knowledge, this is the first study to look at associations between variants in the toll-like receptor family and toll-like receptor levels on monocytes.

## Introduction

Monocytes and macrophages are integral components in the initiation and progression of atherosclerosis (Swirski et al. 2009). Recent work in mice and humans has identified the importance of surface receptor levels in regulating cellular function (Geissmann et al. 2003; Swirski et al. 2009). In humans, monocytes are currently categorized based on their expression of surface receptors, CD14 and CD16. Monocytes expressing CD14 have been labeled inflammatory monocytes, while monocytes expressing CD16 have been labeled resident monocytes. Inflammatory monocytes express additional surface receptors that respond to inflammatory chemokines, including toll-like receptors (TLRs) and myeloperoxidase (MPO). Specifically, the TLR4/nuclear factor-κB pathway has been implicated in inflammatory plaque activation by lipids and plaque destabilization (Kiechl et al. 2003).

CD14 is a potent cofactor enhancing the recognition of TLR2 and TLR4 ligands and high levels of CD14 were associated with increased risk of cardiovascular events and death in dialysis patients (Heine et al. 2008), intima media thickness (IMT; Ulrich et al. 2008), and myocardial infarction (MI; Ozdogru et al. 2007). TLRs mediate the synthesis of proinflammatory cytokines and are upregulated in atherosclerotic lesions (Schoneveld et al. 2008) and in animal models of atherosclerosis (Li and Sun 2007; Mullick et al. 2005; Schoneveld et al. 2005). Studies utilizing human artery tissue have shown significant elevations of TLR1, TLR2, and TLR4 levels in atherosclerotic lesions compared to normal arteries (Edfeldt et al. 2002). Patients with acute MI and unstable angina had higher levels of circulating TLR4 compared to those with stable angina and controls (Ishikawa et al. 2008; Methe et al. 2005). Higher TLR4 levels are also associated with heart failure after MI (Satoh et al. 2006) and surface levels of TLR2 and TLR4 are increased in type 1 diabetic patients (Devaraj et al. 2008). Increased TLR4 expression was also observed at the site of plaque rupture (Ishikawa et al. 2008). Furthermore, recent evidence suggests that variants in TLR genes may be associated with IMT, although the data are inconclusive (Hernesniemi et al. 2008; Labrum et al. 2007; Netea et al. 2004). MPO is a peroxidase in the cytoplasm that breaks down hydrogen peroxide (Klebanoff 2005). Circulating monocytes contain MPO but lose expression in the transition to a macrophage. Higher circulating levels of MPO have been associated with the presence of coronary artery disease (CAD; Zhang et al. 2001) and increased risk of subsequent coronary events in patients with acute coronary syndromes (Baldus et al. 2003).

Despite progress in identifying the importance of cell surface receptors as key regulators of cell function, the field has been partly limited by the lack of conservation of antigens between mouse and humans. Specifically, CD14 and CD16 do not exist in the mouse. Additionally, the flow cytometry methods used to measure these markers are expensive and labor intensive, and require highly skilled personnel. Thus, few large-scale studies have employed this technique. Therefore, little is known about the influence of genetic variants on levels of these markers in monocytes. Thus, insight into the regulation of the levels of these surface receptors is important. We propose to test the hypothesis that the variants in the structural genes for *CD14*, *TLR2*, *TLR4*, and *MPO* were associated with altered monocyte surface receptor levels of these proteins in a biracial cohort of adults from the Atherosclerosis Risk in Communities (ARIC) Carotid MRI Study.

# Methods

## Subjects

The study sample consisted of 2,066 members of the ARIC Study who participated in the ARIC Carotid MRI Study in 2004–2005. ARIC is a cohort study of cardiovascular disease that recruited 15,792, men and women, mainly blacks and whites, from four US communities: Forsyth County, NC; Jackson, MS; suburban Minneapolis, MN; and Washington County, MD. The ARIC Study has been described in detail elsewhere (1989).

In 2004–2005, ARIC participants from the four communities were recruited to participate in the Carotid MRI Study under a stratified sampling design. The sampling goal was to recruit 1,200 participants with high values of maximum carotid artery IMT, and 800 individuals randomly sampled from the remainder of the carotid IMT distribution. The high values of IMT were those above field-center-specific cut points, which were adjusted over the recruitment period to achieve the sampling goal. Maximum IMT was based on measurements over six sites, left and right, common, bifurcation, and internal at each subject's last ARIC ultrasound examination in the 1990s. A total of 4,307 ARIC cohort members were contacted for study, of which 1,404 refused, 837 were ineligible to undergo MRI, and 2,066 whites and blacks were examined. This study was approved by the institutional review board at each field center and all subjects gave informed consent.

## Measurements

A fasting whole blood sample was collected into Cyto-Chex® BCT vacutainer tubes (Streck, Omaha, NE). The BCT tubes were inverted eight times, stored briefly at room temperature, and shipped overnight in temperature-stabilizing packages to the flow cytometry laboratory at the University of Texas Health Science Center at Houston. The containers had sufficient insulating capacity to maintain the transportation temperatures of the specimens. Immediately upon receipt, the laboratory prepared and analyzed samples within 24 h of blood drawing using a Coulter® Epics™XL™ flow cytometer (Beckman Coulter, Inc., Miami, FL). The protocols, antibodies, and the reproducibility of the methods are described in detail elsewhere (Catellier et al. 2008). Briefly, flow cytometry measures fluorescence per cell using the principles of light scattering, light excitation, and emission of fluorochrome molecules. The flow cytometry measures for the ARIC Carotid MRI Study included platelet and monocyte markers, myeloperoxidase, and platelet aggregates with monocytes that were shown in preliminary studies to be reproducible. Given our aim of investigating monocyte activation, the variables for this analysis were restricted to the markers of monocyte activation and included myeloperoxidase (MPO); toll-like receptors (TLR) 2 and 4; CD14 (lipopolysaccharide, LPS, receptor); P-selectin glycoprotein ligand-1 (PSGL-1, CD162); and pan-leukocyte marker (CD45). Data are presented as proportions of cells expressing antigen (%) and/or the relative levels of antigen levels assessed by the median fluorescence intensity (MFI). Coefficients of variation (CV) for both % of positive cells and MFI are shown in Table 1 for replicate samples drawn from a single venipuncture ($n$ = 103). Values range from 2.0 to 9.5. Complete results for intra-individual variability and reliability of these measures in the ARIC Carotid MRI Study have been previously published (Catellier et al. 2008).

Height was measured while participants were standing without shoes, heels together against a vertical mounted ruler. A Detecto Platform Balance was used to measure weight. BMI was calculated as weight (kg)/height$^2$ (m$^2$). Waist circumferences were measured at the level of the umbilicus with the participant standing erect. Plasma total cholesterol and triglycerides were measured by enzymatic methods and HDL cholesterol after dextran sulfate–

magnesium precipitation of non-HDL lipoproteins. LDL cholesterol was calculated via the Friedewald equation.

### Genotyping

For this study, we evaluated tagSNPs in four structural genes, *CD14*, *MPO*, *TLR2*, and *TLR4*, corresponding to the flow cytometry measurements available. TagSNPs within *CD14*, *TLR2*, *TLR4*, and *MPO* were derived using the Haploview Program based on two sources of SNPs: the Caucasian (CEU) and Yoruban (YRI) population from the International HapMap project. The data were analyzed in a race-specific manner on a gene by gene basis using the gene definitions provided by the HapMap database. Known replicate samples were included in the plate set for genotyping and concordance of these duplicated samples for all SNPs was 99.998. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

### Statistical analysis

We excluded those missing all flow cytometry data, missing all SNP data ($n = 166$ for both groups), taking chemotherapy or steroids ($n = 75$) or not consenting to use of DNA ($n = 8$), which left 1,817 for study. All analyses were based on methods suitable for stratified random sample methods, with samples weighted by the inverse of the sampling fractions in the eight strata (4 communities × 2 IMT groups). The sample fractions were based on those persons actually screened for participation. Those who participated (non-refusing eligible) were analyzed as a sub-population (domain) of those invited in calculating variances and confidence intervals of estimators as well as in modeling. Basic statistics of flow cytometry phenotypes were calculated by race groups, using SAS version 9.1. Genotype frequencies were estimated using SUDAAN CROSSTAB, and allele frequencies were calculated from genotype frequencies, as P(AA) + P(Aa)/2 for allele A, where P(AA) and P(Aa) are the weighted frequencies of AA and Aa. Hardy–Weinberg equilibrium was tested using weighted genotype frequencies and their covariance matrix, applying the delta method to get the test statistic. Means of flow cytometry measurements from different genotypes were further evaluated by linear models, using SUDAAN, with appropriate tests for the significance of differences in means among genotype groups. Linear regression and testing were carried out separately for each combination of race and SNP. Different linear models were implemented to estimate: (1) unadjusted means; (2) means adjusted for age and sex; (3) and means adjusted for age, sex, triglycerides, HDL, LDL, waist, BMI, use of cholesterol lowering medications, and current and former smoking status. SNPs with a race-specific minor allele frequency < 1% were not analyzed in that race, which eliminated four race-SNP combinations from the analysis. Minor allele homozygotes and heterozygotes were combined when the number of subjects in a race-specific geno-type group was <10. To correct for multiple comparisons, we used Bonferroni corrections based on the total number of race-SNP combinations (2 races × 15 SNPs + 1 race × 4 SNPs) and set a threshold of $p <$ 0.001 (0.05/34) to deter mine statistical significance. *TLR4* SNPs rs4986790 and rs4986791 affect the extracellular domain of the receptor (Arbour et al. 2000; Lorenz et al. 2001). To investigate the co-segregation of two functional SNPs in *TLR4* (rs4986790 and rs4986791), in whites we compared the following genotype combinations for rs4986790 and rs498691, respectively, AA/CC and AG or GG/CT and in blacks AA/CC and AG or GG/CC.

## Results

Baseline characteristics are shown in Table 2 by race. Differences by race and sex for the monocyte phenotypes have been described elsewhere (Folsom et al. 2009). Allele frequencies for *CD14*, *MPO*, *TLR2*, and *TLR4* are listed in Table 3 including the assessment of HWE. The description of each SNP is listed in Online Table 1. Online Tables 2–21

present unadjusted means for monocyte phenotypes by race as the adjustment for sex did not materially alter the associations. Furthermore, unless otherwise noted, the addition of other covariates also did not materially change the associations.

*TLR2* rs1816702 genotype was significantly associated with CD14+/TLR2+ (% and MFI) in whites, Table 4, but not in blacks ($p > 0.4$, Online Table 3). Specifically, the presence of the minor T-allele was associated with increased receptor levels on the surface of the monocytes. This SNP was not associated with any of the covariates tested in whites (data not shown). However, adjustment for lipids, waist circumference, BMI, and smoking strengthened the association. In blacks, *TLR4* rs5030719 was significantly associated with CD14+/TLR4+ monocytes (MFI) with mean ± SE intensities of 16.7 ± 0.05 and 16.0 ± 0.14 for GG and GT/TT genotypes, respectively (<0.001, Table 5). A similar pattern was seen with the presence of the T-allele associated with lower mean percentage of positive cells, albeit not significant ($p = 0.05$). In whites, rs5030719 is monomorphic.

Two other SNPs appear to be associated with monocyte phenotypes although neither met the a priori defined significance threshold. *TLR4* rs4986790 was associated with CD14+/TLR4+ cells (%) in blacks with means of 16.7 ± 0.06 for AA and 16.4 ± 0.11 for AG/GG genotypes ($p = 0.004$), Online Table 10. *MPO* rs28730837 was associated with monocyte levels of MPO (MFI) in whites with mean intensities of 90.2 ± 0.80 and 79.9 ± 3.59 in CC and CT/TT genotypes, respectively $p = 0.005$), Online Table 21. In blacks, *MPO* rs8067377 was associated with monocyte levels of MPO (MFI) with mean intensities of 104.2 ± 1.8 and 93.0 ± 3.8 in CC and CT/TT genotypes, respectively ($p = 0.008$), Online Table 20. No significant differences were observed for the genotype combinations of rs4986790 and rs498691, Online Tables 22–23.

## Discussion

The major findings of this population-based study were that genetic variants in *TLR2* and *TLR4* were associated with monocyte surface receptor levels of TLR2 and TLR4, respectively, in a biracial cohort of adults. Moreover, these associations between genotype and phenotype were unique to whites (in the case of TLR2) and blacks (in the case of TLR4). This study takes the first step in identifying genetic determinants of surface receptor levels. Given the economic and technical issues related to measuring these phenotypes in large scale studies, identifying genetic variants related to phenotypic levels may enhance our ability to investigate these cellular pathways and atherosclerosis. Epidemiologic studies investigating potential associations between bacterial infection and atherosclerosis provide the initial evidence linking TLR activation and vessel disease (Kalayoglu et al. 2002). In this investigation, we report that *TLR2* rs1816702 and *TLR4* rs5030719 were associated with monocyte phenotypes in whites and blacks, respectively.

*TLR2* rs1816702 is located within intron 2 and is in tight linkage disequilibrium (LD) with rs4696483, also located in intron 2 ($r^2 = 0.93$ HapMap CEU Data). The T-allele was less common in whites ($f = 0.11$) compared to blacks ($f = 0.42$). In whites, the T-allele was associated with an increased percentage of monocytes positive for TLR2. The biological effect of enhanced TLR2 levels in a greater percentage of monocytes positive for TLR2 is not known. In animal models, *TLR2*−/− *ApoE* −/− mice exhibited decreased atherosclerosis, and reduced monocyte chemoattractant protein-1 (MCP-1) production upon stimulation of the TLR2 ligand (Liu et al. 2008). *TLR2* −/− on a *LDLr*−/− background also exhibited decreased atherosclerosis (Mullick et al. 2005). These studies suggest that TLR2 is atherogenic. However, controversy exists as to the role of TLR2 in the monocytes in these studies. Previous genetic work in humans suggested that a frequent *TLR2* SNP, rs5743708 (Arg753Gln), resulted in a non-functional receptor. Patients with the G allele were protected

against restenosis compared to A-carriers (Hamann et al. 2005). Future studies will be needed in both murine models and humans to better understand the role of TLR2 within different cell types and how risk alleles may alter the biology of TLR2 structure and function.

*TLR4* rs5030719 encodes a glutamine to histidine substitution in exon 3 and was only polymorphic in blacks (T-frequency = 0.03). Carriers of the minor T-allele at this site had lower levels of CD14+/TLR4+ cells compared to homozygotes for the wild-type allele. *TLR4* rs4986790 encodes an aspartic acid to glycine substitution in exon 3 and the minor G allele was modestly associated with lower levels of CD14+/TLR4+ monocytes (MFI) ($p$ = 0.004 in blacks and 0.04 in whites). However, we did not observe a relationship with genotype combinations of these two SNPs with monocyte levels. *TLR4* rs4986790 is in moderate LD with rs5030719 ($r^2$ = 0.49 HapMap Yoruba data). Previous work investigating atherosclerotic phenotypes and *TLR4* polymorphisms has focused on rs4986790 and a co-segregating variant that was rare (frequency < 1%) in blacks in our study sample (rs4986791, Thr399Ile). Both *TLR4* rs4986790 and rs4986791 are functional variants that affect the extracellular domain of the receptor (Arbour et al. 2000; Lorenz et al. 2001). *TLR4* rs4986790 has been reported to be associated with carotid artery elasticity (Hernesniemi et al. 2008) and both increased and decreased IMT (Kiechl et al. 2002), albeit inconsistently for the later (Hernesniemi et al. 2008; Norata et al. 2005). The majority of studies found a positive effect of the minor G-allele attributed to an attenuated inflammatory response, corroborating our finding of reduced receptor levels (Hernesniemi et al. 2008; Kiechl et al. 2002). Enquobahrie et al. (2008) reported that *TLR4* haplotypes were associated with MI risk and specifically the minor allele of rs1927911 was associated with a 12% lower MI risk. In aggregate, these results, including our novel finding with TLR4+ monocyte levels, suggest that the *TLR4* gene seems to play a role in the immune response influencing atherosclerosis. Work from the lab of Alan Tall and others have found that dysregulation of cholesterol efflux from macrophages alters the inflammatory profile in response to TLR ligands (Sun et al. 2009; Yvan-Charvet et al. 2010a, b, c; Yvan-Charvet et al. 2008). These findings suggest that functional variants in TLR4 may be important in mediating inflammatory responses to cholesterol and TLR ligands. Furthermore, these results consistently find beneficial effects of minor alleles on atherosclerosis phenotypes, possibly the result of attenuated function of the protein leading to a diminished immune response. Animal models support that this assertion as deficiency in TLR4 is associated with reductions in aortic atherosclerosis (Michelsen et al. 2004), lesion formation (Vink et al. 2002), and arterial remodeling (Hollestelle et al. 2004).

*MPO* rs28730837 encodes an alanine to valine substitution in exon 6 and the minor allele (T) was modestly associated in whites with lower levels of MPO in monocytes ($p$ = 0.005). This SNP was monomorphic in blacks. Conversely, rs8067377, an intronic SNP, was associated in blacks and monomorphic in whites. However, again we see lower levels of MPO levels in monocytes associated with the minor allele ($p$ = 0.008). Previously published *MPO* gene variant studies have focused primarily on a promoter SNP (−463G/A) not genotyped in our study. This SNP has been associated with carotid artery IMT (Makela et al. 2008) and in giant cell arteritis (Salvarani et al. 2008).

Differences in the genetic variation across populations in these immune signaling genes may explain the population-specific results observed. Mounting evidence suggests that evolutionary pressure due to infectious disease is the driving force of population differences in TLR variants (Ferwerda et al. 2007, 2008; Velez et al. 2009, 2010). For *TLR2*, numerous functional variants have been identified that differ in frequency by race and have been shown to be associated with susceptibility to infection (Merx et al. 2007; Velez et al. 2010; Yim et al. 2004). In the case of *TLR4*, the majority of the non-synonymous variation is

located in the third exon that encodes the region of the protein involved in pathogen recognition (Smirnova et al. 2001). Of the four non-synonymous variants in exon 3 genotyped in this study, two were monomorphic in whites. Previous studies of population differences have focused on *TLR4* rs4986790 and rs4986791. In Africans, the minor allele of rs4986790 is relatively common with a frequency of 10–18%, whereas the minor allele of rs4986791 is rare. The rs4986790 variant not only shows an enhanced immune response that increases susceptibility to septic shock (Agnese et al. 2002) but may also protect against mortality in severe malaria (Mockenhaupt et al. 2006). Although these studies did not include the two significant SNPs observed in our study, collectively they provide strong evidence of population-specific differences in gene function.

Despite the novel findings, this study has limitations. First, flow cytometry is a sensitive and reproducible method for detecting receptor surface levels; however, this method is unable to determine if there are changes in the function of the receptor. Secondly, monocytes are produced in the bone marrow and move into the subendothelial space. Thus, measurements in monocyte populations are a moving target and flow cytometry captures only a single snapshot. Finally, in the present analysis, we were able to measure CD14, MPO, TLR2, and TLR4 levels on specific antibody characterized cell populations. Recent work suggests that CD16 may also be an important biomarker (Swirski et al. 2009) but was not measured in our study. Furthermore, CD14+ inflammatory monocytes express high levels of additional surface receptors that were not measured.

In this novel investigation, we identified genetic variants in toll-like receptor genes, *TLR2* and *TLR4*, that were associated with monocyte surface receptor levels of these proteins. Secondly, we identified a modest association with *MPO* variants and MPO cell surface levels in monocytes. Further studies are needed to understand the function of these variants on protein structure and function, the importance of these variants in other populations, and the relationship of genetic variants in these genes and cardiovascular outcome.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The Atherosclerosis Risk in Communities (ARIC) Study is carried out as a collaborative study supported by National Heart, Lung, and Blood Institute contracts N01-HC-55015, N01-HC-55016, N01-HC-55018, N01-HC-55019, N01-HC-55020, N01-HC-55021, and N01-HC-55022. The ARIC MRI Study was supported by U01-HL075572 from the National Heart, Lung, and Blood Institute. The investigators thank the participants and staff in the ARIC Study for their important contributions.

## References

Agnese DM, Calvano JE, Hahm SJ, Coyle SM, Corbett SA, Calvano SE, Lowry SF. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. J Infect Dis. 2002; 186:1522–1525. [PubMed: 12404174]

Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000; 25:187–191. [PubMed: 10835634]

Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Munzel T, Simoons ML, Hamm CW. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation. 2003; 108:1440–1445. [PubMed: 12952835]

Catellier DJ, Aleksic N, Folsom AR, Boerwinkle E. Atherosclerosis Risk in Communities (ARIC) Carotid MRI flow cytometry study of monocyte and platelet markers: intraindividual variability and reliability. Clin Chem. 2008; 54:1363–1371. [PubMed: 18515256]

Devaraj S, Dasu MR, Rockwood J, Winter W, Griffen SC, Jialal I. Increased toll-like receptor (TLR) 2 and TLR4 expression in monocytes from patients with type 1 diabetes: further evidence of a proinflammatory state. J Clin Endocrinol Metab. 2008; 93:578–583. [PubMed: 18029454]

Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation. 2002; 105:1158–1161. [PubMed: 11889007]

Enquobahrie DA, Smith NL, Bis JC, Carty CL, Rice KM, Lumley T, Hindorff LA, Lemaitre RN, Williams MA, Siscovick DS, Heckbert SR, Psaty BM. Cholesterol ester transfer protein, interleukin-8, peroxisome proliferator activator receptor alpha, and Toll-like receptor 4 genetic variations and risk of incident nonfatal myocardial infarction and ischemic stroke. Am J Cardiol. 2008; 101:1683–1688. [PubMed: 18549840]

Ferwerda B, McCall MB, Alonso S, Giamarellos-Bourboulis EJ, Mouktaroudi M, Izagirre N, Syafruddin D, Kibiki G, Cristea T, Hij-mans A, Hamann L, Israel S, ElGhazali G, Troye-Blomberg M, Kumpf O, Maiga B, Dolo A, Doumbo O, Hermsen CC, Stalenhoef AF, van Crevel R, Brunner HG, Oh DY, Schumann RR, de la Rua C, Sauerwein R, Kullberg BJ, van der Ven AJ, van der Meer JW, Netea MG. TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. Proc Natl Acad Sci USA. 2007; 104:16645–16650. [PubMed: 17925445]

Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, Van der Meer JW, Netea MG. Functional consequences of toll-like receptor 4 polymorphisms. Mol Med. 2008; 14:346–352. [PubMed: 18231573]

Folsom AR, Aleksic N, Sanhueza A, Boerwinkle E. Risk factor correlates of platelet and leukocyte markers assessed by flow cytometry in a population-based sample. Atherosclerosis. 2009; 205(1): 272–278. [PubMed: 19124123]

Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003; 19:71–82. [PubMed: 12871640]

Hamann L, Gomma A, Schroder NW, Stamme C, Glaeser C, Schulz S, Gross M, Anker SD, Fox K, Schumann RR. A frequent toll-like receptor (TLR)-2 polymorphism is a risk factor for coronary restenosis. J Mol Med. 2005; 83:478–485. [PubMed: 15875151]

Heine GH, Ulrich C, Seibert E, Seiler S, Marell J, Reichart B, Krause M, Schlitt A, Kohler H, Girndt M. CD14(++)CD16+ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. Kidney Int. 2008; 73:622–629. [PubMed: 18160960]

Hernesniemi JA, Raitakari OT, Kahonen M, Juonala M, Hutri-Kahonen N, Marniemi J, Viikari J, Lehtimaki T. Toll-like receptor 4 gene (Asp299Gly) polymorphism associates with carotid artery elasticity. The cardiovascular risk in young Finns study. Atherosclerosis. 2008; 198:152–159. [PubMed: 17996871]

Hollestelle SC, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A, Strijder CF, Van Middelaar BJ, Pasterkamp G, Quax PH, De Kleijn DP. Toll-like receptor 4 is involved in outward arterial remodeling. Circulation. 2004; 109:393–398. [PubMed: 14699006]

Ishikawa Y, Satoh M, Itoh T, Minami Y, Takahashi Y, Akamura M. Local expression of Toll-like receptor 4 at the site of ruptured plaques in patients with acute myocardial infarction. Clin Sci (Lond). 2008; 115:133–140. [PubMed: 18282141]

Kalayoglu MV, Libby P, Byrne GI. Chlamydia pneumoniae as an emerging risk factor in cardiovascular disease. JAMA. 2002; 288:2724–2731. [PubMed: 12460096]

Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, Schwartz DA. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med. 2002; 347:185–192. [PubMed: 12124407]

Kiechl S, Wiedermann CJ, Willeit J. Toll-like receptor 4 and atherogenesis. Ann Med. 2003; 35:164–171. [PubMed: 12822738]

Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005; 77:598–625. [PubMed: 15689384]

Labrum R, Bevan S, Sitzer M, Lorenz M, Markus HS. Toll receptor polymorphisms and carotid artery intima-media thickness. Stroke. 2007; 38:1179–1184. [PubMed: 17332447]

Li H, Sun B. Toll-like receptor 4 in atherosclerosis. J Cell Mol Med. 2007; 11:88–95. [PubMed: 17367503]

Liu X, Ukai T, Yumoto H, Davey M, Goswami S, Gibson FC 3rd, Genco CA. Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids. Atherosclerosis. 2008; 196:146–154. [PubMed: 17466307]

Lorenz E, Frees KL, Schwartz DA. Determination of the TLR4 genotype using allele-specific PCR. Biotechniques. 2001; 31:22–24. [PubMed: 11464514]

Makela R, Loimaala A, Nenonen A, Mercuri M, Vuori I, Huhtala H, Oja P, Bond G, Koivula T, Lehtimaki T. The association of myeloperoxidase promoter polymorphism with carotid atherosclerosis is abolished in patients with type 2 diabetes. Clin Biochem. 2008; 41:532–537. [PubMed: 18280811]

Merx S, Neumaier M, Wagner H, Kirschning CJ, Ahmad-Nejad P. Characterization and investigation of single nucleotide polymorphisms and a novel TLR2 mutation in the human TLR2 gene. Hum Mol Genet. 2007; 16:1225–1232. [PubMed: 17409197]

Methe H, Kim JO, Kofler S, Weis M, Nabauer M, Koglin J. Expansion of circulating Toll-like receptor 4-positive monocytes in patients with acute coronary syndrome. Circulation. 2005; 111:2654–2661. [PubMed: 15883205]

Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M. Lack of Toll-like receptor 4 or myeloid diVerentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deWcient in apolipoprotein E. Proc Natl Acad Sci USA. 2004; 101:10679–10684. [PubMed: 15249654]

Mockenhaupt FP, Cramer JP, Hamann L, Stegemann MS, Eckert J, Oh NR, Otchwemah RN, Dietz E, Ehrhardt S, Schroder NW, Bienzle U, Schumann RR. Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. Proc Natl Acad Sci USA. 2006; 103:177–182. [PubMed: 16371473]

Mullick AE, Tobias PS, Curtiss LK. Modulation of atherosclerosis in mice by Toll-like receptor 2. J Clin Invest. 2005; 115:3149–3156. [PubMed: 16211093]

Netea MG, Hijmans A, van Wissen S, Smilde TJ, Trip MD, Kullberg BJ, de Boo T, Van der Meer JW, Kastelein JJ, Stalenhoef AF. Toll-like receptor-4 Asp299Gly polymorphism does not inXuence progression of atherosclerosis in patients with familial hypercholesterolaemia. Eur J Clin Invest. 2004; 34:94–99. [PubMed: 14764071]

Norata GD, Garlaschelli K, Ongari M, Raselli S, Grigore L, Benvenuto F, Maggi FM, Catapano AL. EVect of the Toll-like receptor 4 (TLR-4) variants on intima-media thickness and monocyte-derived macrophage response to LPS. J Intern Med. 2005; 258:21–27. [PubMed: 15953129]

Ozdogru I, Inanc MT, Eryol NK, Dogan A, Kaya MG, Gul I, Kalay N. CD14+ monocyte levels in subgroups of acute coronary syndromes. Coron Artery Dis. 2007; 18:519–522. [PubMed: 17925604]

Salvarani C, Casali B, Farnetti E, Pipitone N, Nicoli D, Macchioni PL, Cimino L, Bajocchi GL, Catanoso MG, Pattacini L, Ghinoi A, Restuccia G, Boiardi L. −463 G/A myeloperoxidase promoter polymorphism in giant cell arteritis. Ann Rheum Dis. 2008; 67:485–488. [PubMed: 17704068]

Satoh M, Shimoda Y, Maesawa C, Akatsu T, Ishikawa Y, Minami Y, Hiramori K, Nakamura M. Activated toll-like receptor 4 in monocytes is associated with heart failure after acute myocardial infarction. Int J Cardiol. 2006; 109:226–234. [PubMed: 16051384]

Schoneveld AH, Oude Nijhuis MM, van Middelaar B, Laman JD, de Kleijn DP, Pasterkamp G. Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development. Cardiovasc Res. 2005; 66:162–169. [PubMed: 15769459]

Schoneveld AH, Hoefer I, Sluijter JP, Laman JD, de Kleijn DP, Pasterkamp G. Atherosclerotic lesion development and Toll like receptor 2 and 4 responsiveness. Atherosclerosis. 2008; 197:95–104. [PubMed: 17888930]

Smirnova I, Hamblin MT, McBride C, Beutler B, Di Rienzo A. Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans. Genetics. 2001; 158:1657–1664. [PubMed: 11514453]

Sun Y, Ishibashi M, Seimon T, Lee M, Sharma SM, Fitzgerald KA, Samokhin AO, Wang Y, Sayers S, Aikawa M, Jerome WG, Ostrowski MC, Bromme D, Libby P, Tabas IA, Welch CL, Tall AR. Free cholesterol accumulation in macrophage membranes activates Toll-like receptors and p38 mitogen-activated protein kinase and induces cathepsin K. Circ Res. 2009; 104:455–465. [PubMed: 19122179]

Swirski FK, Weissleder R, Pittet MJ. Heterogeneous in vivo behavior of monocyte subsets in atherosclerosis. Arterioscler Thromb Vasc Biol. 2009; 29(10):1424–1432. [PubMed: 19372462]

The Atherosclerosis Risk in Communities (ARIC). Study: design and objectives. The ARIC investigators. Am J Epidemiol. 1989; 129(4):687–702. [PubMed: 2646917]

Ulrich C, Heine GH, Gerhart MK, Kohler H, Girndt M. Proinflammatory CD14+CD16+ monocytes are associated with sub-clinical atherosclerosis in renal transplant patients. Am J Transpl. 2008; 8:103–110.

Velez DR, Hulme WF, Myers JL, Weinberg JB, Levesque MC, Stryjewski ME, Abbate E, Estevan R, Patillo SG, Gilbert JR, Hamilton CD, Scott WK. NOS2A, TLR4, and IFNGR1 interactions inXuence pulmonary tuberculosis susceptibility in African-Americans. Hum Genet. 2009; 126:643–653. [PubMed: 19575238]

Velez DR, Wejse C, Stryjewski ME, Abbate E, Hulme WF, Myers JL, Estevan R, Patillo SG, Olesen R, Tacconelli A, Sirugo G, Gilbert JR, Hamilton CD, Scott WK. Variants in toll-like receptors 2 and 9 inXuence susceptibility to pulmonary tuberculosis in Caucasians, African-Americans, and West Africans. Hum Genet. 2010; 127:65–73. [PubMed: 19771452]

Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, Quax PH, Lim SK, Borst C, Pasterkamp G, de Kleijn DP. In vivo evidence for a role of toll-like receptor 4 in the development of intimal lesions. Circulation. 2002; 106:1985–1990. [PubMed: 12370224]

Yim JJ, Ding L, SchaVer AA, Park GY, Shim YS, Holland SM. A microsatellite polymorphism in intron 2 of human Toll-like receptor 2 gene: functional implications and racial diVerences. FEMS Immunol Med Microbiol. 2004; 40:163–169. [PubMed: 14987735]

Yvan-Charvet L, Welch C, Pagler TA, Ranalletta M, LamkanW M, Han S, Ishibashi M, Li R, Wang N, Tall AR. Increased inXammatory gene expression in ABC transporter-deWcient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil inWltration of atherosclerotic lesions. Circulation. 2008; 118(18):1837–1847. [PubMed: 18852364]

Yvan-Charvet L, Kling J, Pagler T, Li H, Hubbard B, Fisher T, Sparrow CP, Taggart AK, Tall AR. Cholesterol eZux potential and antiinXammatory properties of high-density lipoprotein after treatment with niacin or anacetrapib. Arterioscler Thromb Vasc Biol. 2010a; 30(7):1430–1438. [PubMed: 20448206]

Yvan-Charvet L, Pagler TA, Seimon TA, Thorp E, Welch CL, Witztum JL, Tabas I, Tall AR. ABCA1 and ABCG1 protect against oxidative stress-induced macrophage apoptosis during eVerocytosis. Circ Res. 2010b; 106(12):1861–1869. [PubMed: 20431058]

Yvan-Charvet L, Wang N, Tall AR. Role of HDL, ABCA1, and ABCG1 transporters in cholesterol eZux and immune responses. Arterioscler Thromb Vasc Biol. 2010c; 30(2):139–143. [PubMed: 19797709]

Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, Topol EJ, Sprecher DL, Hazen SL. Association between myeloperoxidase levels and risk of coronary artery disease. JAMA. 2001; 286:2136–2142. [PubMed: 11694155]

## Table 1

Coefficients of variation (CV) for replicate samples drawn from a single venipuncture ($n$ = 103 replicate pairs)

| Monocyte markers | Alternate name | CV | |
|---|---|---|---|
| | | % | MFI |
| CD14 | Lipopolysaccharide (LPS) receptor | | 3.7 |
| CD14/TLR2 | Toll-like receptor-2 | 4.4 | 2.0 |
| CD14/TLR4 | Toll-like receptor-4 | 2.0 | 2.6 |
| CD14/CD41/TLR4 | GPIIb ($\alpha_{IIb}$) | 9.5 | 6.9 |
| CD14/CD162 | P-selectin glycoprotein ligand-1 (PSGL-1) | | 3.3 |
| CD14/TLR2/CD162 | | 4.3 | 2.0 |
| CD14/COX-2 | | 1.3 | 7.8 |
| CD45 | Pan-leukocyte marker | | 3.8 |
| CD45/MPO | Myeloperoxidase | | 8.3 |

## Table 2

Baseline characteristics of subjects genotyped by racial group (means ± SD or percentage)

| Variable | Blacks | Whites | *p* value |
|---|---|---|---|
| Male (%) | 36 | 47 | 0.001 |
| Age (years) | 69 ± 5 | 71 ± 5 | <0.001 |
| Monocyte phenotypes | | | |
| CD14+ (MFI) | 120 ± 22 | 109 ± 20 | <0.001 |
| CD14+/TLR2+ (%) | 56 ± 10 | 64 ± 12 | <0.001 |
| CD14+/TLR2+ (MFI) | 13 ± 0.80 | 14 ± 1.5 | <0.001 |
| CD14+/TLR4+ (%) | 64 ± 3.3 | 65 ± 4.2 | <0.001 |
| CD14+/TLR4+ (MFI) | 17 ± 0.92 | 16 ± 1.0 | <0.001 |
| CD14+/CD41+/TLR4+ (%) | 12 ± 2.6 | 11 ± 2.7 | <0.001 |
| CD14+/CD41+/TLR4+ (MFI) | 49 ± 6.6 | 46 ± 5.8 | <0.001 |
| CD14+/CD162+ (MFI) | 114 ± 14 | 111 ± 15 | 0.001 |
| CD14+/TLR2+/CD162+ (%) | 57 ± 10 | 65 ± 11 | <0.001 |
| CD14+/TLR2+/CD162+ (MFI) | 13 ± 0.81 | 14 ± 1.7 | <0.001 |
| CD14+/COX2+ (%) | 98 ± 2.7 | 98 ± 3.6 | 0.60 |
| CD14+/COX2+ (MFI) | 17 ± 2.9 | 17 ± 2.8 | 0.31 |
| CD45+ (MFI) | 71 ± 9.8 | 76 ± 10 | <0.001 |
| CD45+/MPO+ (MFI) | 102 ± 28 | 17 ± 2.8 | <0.001 |
| Triglycerides (mg/dL) | 127 ± 61 | 158 ± 90 | <0.001 |
| HDL cholesterol (mg/dL) | 51 ± 14 | 50 ± 15 | 0.122 |
| LDL cholesterol (mg/dL) | 124 ± 36 | 112 ± 35 | <0.001 |
| Waist circumference (cm) | 106 ± 15 | 100 ± 14 | <0.001 |
| BMI ($kg/m^2$) | 31 ± 6 | 29 ± 5 | <0.001 |
| Current smoking (% yes) | 10 | 7 | 0.009 |

## Table 3

Allele frequencies calculated using weighted counts by racial group (%)

| SNP (allele) | Blacks | Whites |
|---|---|---|
| *CD14* | | |
| rs4914 (G) | 7 | 12 |
| *TLR2* | | |
| rs1816702 (T) | 42 | 11 |
| rs1898830 (G) | 11* | 35 |
| rs3804099 (T) | 41 | 56 |
| rs3804100 (C) | 6 | 7 |
| rs11938228 (A) | 13 | 37 |
| *TLR4* | | |
| rs1927907 (A) | 28* | 15 |
| rs1927911 (C) | 37 | 73 |
| rs4986790 (G) | 8 | 6 |
| rs4986791 (T) | 0.9 | 7 |
| rs5030717 (G) | 18 | 12 |
| rs12344353 (C) | 14 | 6 |
| rs5030718 (A) | 3 | 0 |
| rs5030719 (T) | 3 | 0 |
| rs5030728 (A) | 13 | 31 |
| rs11536869 (G) | 0.3 | 4* |
| *MPO* | | |
| rs2071409 (C) | 14 | 15 |
| rs7208693 (A) | 18 | 8* |
| rs8067377 (T) | 9 | 0.1 |
| rs28730837 (T) | 0.3 | 2* |

*Not in Hardy–Weinberg Equilibrium ($p < 0.05$)

**Table 4**

Means and standard errors of monocyte TLR2+ markers, percent of positive cells (%) and median fluorescence intensity (MFI), by *TLR2* rs1816702 in whites

| Monocyte markers | Unit | CC (*n* = 1048) | CT and TT (*n* = 306) | *p* value | | |
|---|---|---|---|---|---|---|
| | | | | Unadjusted | Age and sex adjusted | Maximally adjusted[a] |
| CD14+/TLR2+/CD162+ | % | 64.3 ± 0.42 | 68.7 ± 0.97 | 0.00003 | 0.00003 | 0.00002 |
| | MFI | 13.8 ± 0.04 | 14.4 ± 0.23 | 0.01 | 0.01 | 0.00005 |
| CD14+/TLR2+ | % | 63.0 ± 0.42 | 67.4 ± 0.98 | 0.00003 | 0.00003 | 0.00002 |
| | MFI | 14.0 ± 0.04 | 14.6 ± 0.22 | 0.008 | 0.008 | 0.00001 |

[a]Age, sex, triglycerides, HDL, LDL, waist, BMI and smoking status, and lifetime smoking exposure

## Table 5

Unadjusted means and standard errors of TLR4+ markers, percent of positive cells (%) and median fluorescence intensity (MFI), by *TLR4* rs5030719 in blacks

| Monocyte markers | Unit | GG (*n* = 409) | GT/TT (*n* = 21) | Unadjusted *p* value |
|---|---|---|---|---|
| CD14+/CD41+/TLR4+ | % | 11.7 ± 0.15 | 11.3 ± 0.51 | 0.44 |
| | MFI | 48.6 ± 0.40 | 49.2 ± 1.64 | 0.70 |
| CD14+/TLR4+ | % | 64.2 ± 0.20 | 63.0 ± 0.56 | 0.05 |
| | MFI | 16.7 ± 0.05 | 16.0 ± 0.14 | <0.00001 |