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Toll-like receptor 2 and 4 stimulation elicits an enhanced inflammatory response in human obese patients with atherosclerosis

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ABSTRACT

The innate immune response elicited by activation of TLRs (Toll-like receptors) plays an important role in the pathogenesis of atherosclerosis. We hypothesized that cardiovascular risk factors are associated with the activation status of the innate immune system. We therefore assessed the responsiveness of TLRs on circulating cells in two groups of patients with established atherosclerosis and related this to the presence of cardiovascular risk factors. TNF (tumour necrosis factor)-α release induced by TLR2 and TLR4 activation was measured in patients with established coronary [PCI (percutaneous coronary intervention) study, $n = 78$] or carotid artery disease [CEA (carotid endarterectomy) study, $n = 104$], by stimulating whole blood samples with lipopolysaccharide (TLR4 ligand) and Pam_3CSK_4 [tripalmitoylcysteinylseryl-(lysyl)$_4$; TLR2 ligand]. As an early activation marker, CD11b expression was measured by flow cytometry on $CD14^+$ cells. Obesity was the 'only' risk factor that correlated with the TLR response. In both studies, obese patients had significantly higher TNF-α levels after stimulation of TLR2 compared with non-obese patients [16.9 (7.7–49.4) compared with 7.5 (1.5–19.2) pg/ml ($P = 0.008$) in coronary artery disease and 14.6 (8.1–28.4) compared with 9.5 (6.1–15.7) pg/ml ($P = 0.015$) in carotid artery disease; values are medians (interquartile range)]. Similar results were obtained following TLR4 stimulation. The enhanced inflammatory state in obese patients was also confirmed by a significant increased expression of the activation marker CD11b on circulating monocytes. In conclusion, obesity is associated with an enhanced TLR response in patients suffering from established atherosclerotic disease.

Key words: atherosclerosis, cardiovascular disease, innate immunity, obesity, risk factor, Toll-like receptor (TLR).

Abbreviations: apoCIII, apolipoprotein CIII; BMI, body mass index; CEA, carotid endarterectomy; CRP, C-reactive protein; Cy5, indodicarbocyanine; hsCRP, high-sensitive CRP; IL, interleukin; IQR, interquartile range; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MI, myocardial infarction; NEFA, non-esterified 'free' fatty acid; NF-κB, nuclear factor κB; o/n, overnight; Pam_3CSK_4, tripalmitoylcysteinylseryl-(lysyl)$_4$; PCI, percutaneous coronary intervention; PE, phycoerythrin; TLR, Toll-like receptor; TNF, tumour necrosis factor.

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INTRODUCTION

Atherosclerosis is considered as a generalized chronic inflammatory disorder of the vascular tree [1,2]. Patients suffering from chronic inflammatory diseases such as systemic lupus erythematosus or rheumatoid arthritis have an increased risk for developing atherosclerosis [3,4]. A potential clue for the underlying mechanisms of this increased prevalence might be the chronic inflammatory state to which patients are subjected. This enhanced systemic inflammatory state drives the successive activation of endothelial cells, leucocyte adherence and migration through the arterial wall leading to the production of pro-inflammatory cytokines. The relevance of smoking, diabetes, hypercholesterolaemia and hypertension in the development of atherosclerosis is evident [5]. A possible explanation for the increased risk of atherosclerosis, in the presence of these traditional risk factors, is the enhanced activation of the immune system.

Obesity is a worldwide increasing problem, especially in Western societies [6–8]. Obesity is associated with an increased risk of acquiring traditional cardiovascular risk factors, potentiating the development of atherosclerosis and concomitant cardiovascular complications [9–11]. Obesity is also directly related to the development and progression of atherosclerosis [12–15]. Several studies have provided evidence that adipose tissue is not just an energy storage depot but also a source of cytokine-producing inflammatory cells [16–20].

TLRs (Toll-like receptors), which are part of the innate immune system, are pattern-recognizing receptors forming the first line of defence, quickly reacting to evolutionarily conserved pathogens [21]. However, endogenous ligands such as extra domain A, heat-shock proteins, fibronectin and NEFAs (non-esterified 'free' fatty acids) are also able to activate these receptors, resulting into a pro-inflammatory cytokine response. TLRs are found in atherosclerotic tissue but also have shown to play a role in lipid uptake in macrophages and are expressed on circulating monocytes [22,23]. We and others have shown the importance of these receptors in the initiation and progression of atherosclerosis [24–27]. Responsiveness upon ligation of these receptors can be measured in whole blood, *ex vivo*. Previously, we showed that responsiveness altered with different severities of coronary artery stenosis [28]. Others showed that this response was elevated in patients having unstable angina pectoris or acute MI (myocardial infarction) compared with patients with stable angina [29,30].

Considering the central role of the TLR in atherosclerosis development, we hypothesized that cardiovascular risk factors are associated with an altered activation status of the innate immune system, reflected by differences in TLR responsiveness. We assessed the TLR response in patients with established atherosclerosis in two different territories: atherosclerosis of the coronary [PCI (percutaneous coronary intervention) study] and of the carotid artery [CEA (carotid endarterectomy) study] and related this to the presence of cardiovascular risk factors. In addition, we measured expression of the early activation marker CD11b on circulating monocytes.

We demonstrate that obesity is associated with an altered TLR response in patients with established atherosclerosis of the coronary or carotid artery.

MATERIALS AND METHODS

Patients

Two cohorts of patients were analysed for the present study. Both studies were approved by the local medical committee board, and informed consents were obtained.

The first cohort consisted of 100 subsequent patients scheduled for PCI study at the University Medical Centre Utrecht.

The second group consisted of 111 consecutive patients scheduled for CEA study at the University Medical Centre Utrecht and at the St Antonius Hospital, Nieuwegein. This last patient group is part of the Athero-express Biobank study [31].

Clinical data were obtained from patients' medical file records and from preprocedural filled-in questionnaires. Risk factors were scored as follows: hypertension: the use of one or more antihypertensive drugs (>3 months before intervention); hypercholesterolaemia: the use of statins for at least 3 months before intervention; diabetes: the use of anti-diabetic medication (>3 months before intervention); positive family history of heart disease: more than one family member with a cardiac event [MI, AP (angina pectoris) or CABG (coronary artery bypass graft surgery)] <60 years. Obese patients were classified as having a BMI (body mass index) >25.0 and non-obese patients as having a BMI $\leqslant$25.0. Smoking status was derived from the preoperative intake questionnaire of the Department of Anesthesiology.

Blood sampling

PCI study

Whole blood samples were drawn in LH (lithium–heparin) and EDTA-anti-coagulated tubes before PCI. To prevent premature leucocyte activation, all tubes were kept on ice until further processing. The effect of short-term preservation on ice on cytokine production was evaluated before initiation of this study. This short-term preservation did not influence the experiments. A portion (100 μl) of blood was stimulated with 100 μl of the synthetic TLR2 ligand Pam_3CSK_4 [tripalmitoylcysteinylseryl-(lysyl)$_4$; Novabiochem] at 500 ng/ml and 100 μl of the TLR4 ligand LPS (lipopolysaccharide; derived from *Escherichia coli* serotype O55:B5; Sigma) at 10 ng/ml. Samples

were incubated o/n (overnight) at 37 °C and 5 % CO_2. After o/n incubation, samples were centrifuged at 1000 rev./min for 5 min, and supernatants were collected and stored at −20 °C until further processing. As a surrogate of the TLR2 and TLR4 response, TNF (tumour necrosis factor)-α was measured in the supernatants using an ELISA, according to the manufacturer's protocol (Pelikine-compact; Sanquin). TNF-α levels were corrected for white blood cell count, which was measured using a haematology analyser (Celldyn 1700; Abbott Diagnostics).

In EDTA-anti-coagulated blood samples, TLR2, TLR4 and CD11b expression on $CD14^+$ monocytes were determined by flow cytometry (Cytomics FC500; Beckman Coulter). Whole blood samples were stained for CD14 [PE (phycoerythrin)-Cy5 (indodicarbocyanine)] combined with either TLR2 (FITC) and TLR4 (PE) or CD11b (FITC) (all Serotec). TLR2, TLR4 and CD11b expression levels are referred to as MFI (mean fluorescence intensity) on $CD14^+$ monocytes. CRP (C-reactive protein) levels in serum were determined using a Nefelometer (Dade Behring, now Siemens Healthcare Diagnostics), performed by the Clinical Chemistry Laboratory of the University Medical Center Utrecht hospital.

CEA study

Whole blood samples were drawn before incision, immediately after anaesthesia. In a substudy, the effect of anaesthetics was investigated on TLR activation. The anaesthetics did not influence the TNF-α levels (results not shown). Samples were stored in heparinized tubes and immediately processed at the laboratory. Blood samples were stimulated by using the same protocol as used in the PCI study, but, instead of o/n incubation, samples were stimulated for 2 h. As a surrogate of the TLR2 and TLR4 response, TNF-α, IL (interleukin) 6 and IL8 were measured in the supernatants using a multiplex immunoassay system, according to the manufacturer's protocol, (BioPlex 200; Bio-Rad Laboratories). TNF-α, IL6 and IL8 levels were corrected for white blood cell count.

In heparin-anti-coagulated blood samples, TLR2, TLR4 and CD11b expression on $CD14^+$ monocytes were examined by flow cytometry (Cytomics FC500; Beckman Coulter). Whole blood samples were stained for CD14 (RPE-Cy5) combined with TLR2 (FITC) and TLR4 (PE) (Serotec) and CD11b [PE-Cy7 (indotricarbocyanine)] (eBiosciences). TLR2, TLR4 and CD11b expression levels are represented as MFI on $CD14^+$ monocytes. CD11b expression on $CD14^+$ cells was also measured after stimulation. A portion (100 μl) of heparinized whole blood was stimulated with 100 μl of (i) PBS (negative control), (ii) Pam_3CSK_4 (500 ng/ml) (TLR2 stimulation), (iii) LPS (100 ng/ml) (TLR4 stimulation) and (iv) PMA (non-specific stimulation). Stimulated blood samples were incubated for 30 min at 37 °C and 5 % CO_2.

Exclusions

In the PCI study, 22 patients were excluded for analysis: nine patients were suffering from acute MI within 24 h prior to PCI, 12 patients suffered from chronic inflammatory disorders receiving corticosteroid drugs and, finally, one patient was excluded because information about traditional risk factors was lacking.

In the CEA study, seven patients were excluded for analysis: four patients with a chronic inflammatory disorder receiving corticosteroid drugs preoperatively and three patients because white blood cell counts were not performed.

Statistics

Values are medians [IQR (interquartile range)], unless otherwise stated. TNF-α levels, mean fluorescence levels and CRP levels were not normally distributed. After ln-transformation, the data were normally distributed. Differences between two independent variables were calculated with an independent-sample Student's t test. Differences between three or more independent variables were calculated by using one-way ANOVA. Differences between two dichotomous values were calculated using a χ^2 test. Differences between two continuous variables were calculated by using the Spearman test. A P value < 0.05 was considered as statistically significant. Multivariate correction was performed by using a linear regression model. Gender, age, hypertension, hypercholesterolaemia, diabetes, smoking status, positive family history for cardiovascular disease, hsCRP (high-sensitive CRP) levels and BMI <25 or BMI >25 were analysed with a linear regression model. In the CEA study, hsCRP levels were not available, and a positive family history for cardiovascular disease was left out the model because of missing data from 18 patients. All statistical analyses were performed with PASW statistics version 17 (SPSS).

RESULTS

A total of 22 patients in the PCI study and seven patients in the CEA study were excluded from analysis (for details, see the Materials and methods section), leaving data from 78 (PCI study) and 104 patients (CEA study) used for analysis. Clinical characteristics are depicted in Table 1 and in Supplementary Table S1 (at http://www.clinsci.org/cs/121/cs1210205add.htm).

In both studies, TNF-α was used as a specific marker for a TLR response. Several authors have demonstrated the TLR specificity of this cytokine [32,33]. Stimulation of whole blood samples with PBS did not result in detectable TNF-α levels and, in addition, stimulation

Table 1 Patient characteristics

Values are medians (IQR), or number of patients (percentage). In both groups, obese patients (BMI >25) were significantly younger compared with non-obese patients. $^{*}P < 0.05$ and $^{**}P < 0.001$ between the two groups. ACE, angiotensin-converting enzyme; A2, angiotensin II receptor.

	PCI study			CEA study		
Characteristic	Total ($n = 78$)	BMI <25 ($n = 23$)	BMI >25 ($n = 55$)	Total ($n = 104$)	BMI <25 ($n = 37$)	BMI >25 ($n = 67$)
Gender (male/female) (*n*)	56/22	15/8	41/14	69/35	23/14	46/21
Age (years)	62.5 (52.5–69.7)	65.0 (61.0–72.0)*	58.6 (51.9–67.2)*	70.4 (62.2–81.0)	73.7 (66.3–79.9)*	69.3 (61.7–74.6)*
BMI (kg/m^2)	26.9 (24.6–34.9)	24.0 (22.6–24.7)**	28.7 (26.6–32.4)**	26.2 (24.1–29.7)	23.1 (21.8–24.2)**	29.0 (26.6–30.7)**
Hypertension (*n*)	52 (67%)	13 (57%)	39 (71%)	87 (84%)	30 (81%)	57 (85%)
Diabetes (*n*)	16 (21%)	4 (17%)	12 (22%)	21 (20%)	8 (22%)	13 (19%)
Hypercholesterolaemia (*n*)	72 (92%)	21 (91%)	51 (93%)	83 (80%)	28 (76%)	55 (82%)
Smokers (*n*)	5 (6%)	2 (9%)	3 (6%)	26 (25%)	7 (19%)	19 (28%)
Family history of heart disease <60 years (*n*)	41 (53%)	13 (57%)	28 (51%)	27 (31%)†	8 (30%)†	19 (32%)†
Medication (*n*)						
β-Blocker	73 (94%)	23 (100%)	50 (91%)	50 (48%)	16 (43%)	34 (51%)
Calcium antagonist	24 (31%)	6 (26%)	18 (33%)	24 (23%)	8 (22%)	16 (24%)
Nitrates	37 (47%)	10 (44%)	27 (49%)	11 (11%)	4 (11%)	7 (10%)
ACE inhibitor	19 (24%)	4 (17%)	15 (27%)	33 (32%)	10 (27%)	23 (34%)
A2 antagonist	10 (13%)	4 (17%)	6 (11%)	8 (8%)	3 (8%)	5 (8%)
Diuretics	10 (13%)	2 (9%)	8 (15%)	46 (44%)	13 (35%)	33 (49%)
Anti-arrhythmic	5 (6%)	2 (9%)	3 (6%)	4 (4%)	1 (3%)	3 (5%)
Statin	72 (92%)	21 (91%)	51 (93%)	84 (81%)	28 (76%)	56 (84%)
Dipyridamole/aspirin	69 (89%)	20 (87%)	49 (89%)	62 (60%)	21 (57%)	41 (61%)
Clopidogel	60 (77%)	19 (83%)	41 (75%)	7 (7%)	4 (11%)	3 (4%)
Coumarin	8 (10%)	4 (17%)	4 (7%)	7 (7%)	4 (11%)	3 (4%)

†No data could be obtained concerning family history of heart disease from 18 patients.

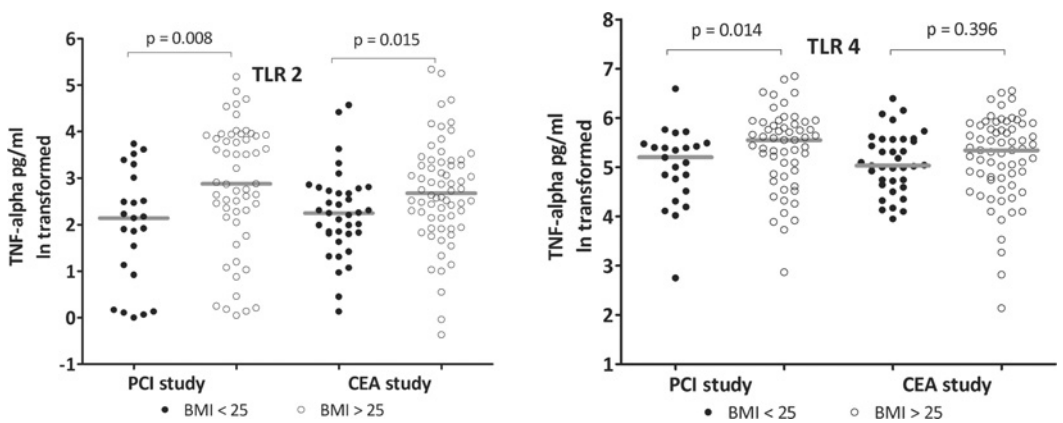

Figure 1 TLR response and obesity

TNF-α levels (ln-transformed) are depicted on the *y*-axis with levels following TLR2 stimulation (with Pam_3CSK_4) in the left-hand panel and levels following TLR4 stimulation (with LPS) in the right-hand panel. (○) Obese patients; (●) non-obese patients. *P* values are adjusted for cardiovascular risk factors.

with the specific TLR2 and TLR4 agonists Pam_3CSK_4 and LPS resulted in a dose-dependent TNF-α release (results not shown).

In both studies, obese patients showed significantly higher TNF-α levels after stimulation of TLR2 compared with non-obese patients [16.9 (7.7–49.4) compared with 7.5 (1.5–19.2) pg/ml (adjusted *P* value = 0.008) and 14.6 (8.1–28.4) compared with 9.5 (6.1–15.7) pg/ml (adjusted *P* value = 0.015) for the PCI and CEA study respectively] (Figure 1). In the PCI study, TNF-α levels in obese patients were also significantly higher compared with non-obese patients following TLR4

Table 2 TNF-α levels following TLR2 stimulation (with Pam_3CSK_4) and TLR4 stimulation (with LPS) and risk factors for atherosclerosis

Values are median (IQR) levels (pg/ml). The number of patients is provided in Table 1. $^{*}P < 0.05$ and $^{**}P < 0.01$ between the two groups.

(a) TLR2 stimulation

	TNF-α (pg/ml) after TLR2 stimulation			
	PCI study		CEA study	
Risk factor	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Hypertension	11 (3–35)	12 (6–22)	18 (8–50)	14 (7–25)
Diabetes	10 (1–26)	14 (6–44)	12 (7–20)	12 (7–25)
Hypercholesterolaemia	12 (4–42)	23 (8–40)	11 (6–22)	15 (9–31)
Family history of heart disease <60 years	15 (6–46)	11 (3–36)	13 (7–32)†	12 (6–19)†
BMI (BMI <25 compared with BMI >25)	7 (2–19)**	17 (8–49)**	9 (6–16)*	15 (8–28)*
Smoking	8 (2–22)	13 (5–43)	11 (6–20)	13 (7–25)
Active smoker compared with past smoker <6 months	8 (2–22)	11 (4–50)	11 (6–20)	7 (4–23)
Past smoker >6 months compared with no smoking ever	14 (3–43)	13 (5–45)	13 (9–22)	16 (10–41)

(b) TLR4 stimulation

	TNF-α (pg/ml) after TLR4 stimulation			
	PCI study		CEA study	
Risk factor	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Hypertension	221 (113–328)	230 (143–317)	175 (102–297)	245 (152–384)
Diabetes	191 (79–218)	239 (128–334)	196 (112–271)	192 (110–317)
Hypercholesterolaemia	223 (113–328)	219 (196–287)	190 (103–302)	202 (146–329)
Family history of heart disease <60 years	217 (114–311)	222 (142–364)	227 (112–340)†	165 (108–297)†
BMI (BMI <25 compared with BMI >25)	181 (91–237)*	257 (129–369)*	154 (105–261)	209 (119–340)
Smoking	94 (54–268)	224 (127–325)	206 (125–312)	185 (111–300)
Active smoker compared with past smoker <6 months	94 (54–268)	257 (168–363)	206 (125–312)	163 (68–303)
Past smoker >6 months compared with no smoking ever	213 (115–314)	243 (159–359)	215 (115–300)	191 (117–316)

†No data could be obtained concerning family history of heart disease from 18 patients; these patients were excluded for this analysis.

stimulation [257.1 (129.0–368.7) compared with 180.9 (91.2–236.6) pg/ml (adjusted P value = 0.014)]. In the CEA study, no significant enhancement was observed [209.4 (118.9–340.2) compared with 154.2 (105.4–260.9) pg/ml (adjusted P value = 0.396)] (Figure 1). In the PCI study, we observed a trend towards lower levels of TNF-α following TLR2 and TLR4 stimulation in patients who were active smokers compared with patients who did not smoke. However, this trend was not observed in the CEA study (Table 2). When taking other risk factors into account, we observed no differences in responsiveness between patients without or with these cardiovascular risk factors, statin or antihypertension medication use (Table 2 and Supplementary Table S2 at http://www.clinsci.org/cs/121/cs1210205add.htm).

In the CEA study, we also quantified the levels of IL6 and IL8 following TLR stimulation. Obese patients also had increased levels of IL6 following TLR2 and TLR4 stimulation compared with non-obese patients [7 (3–30) compared with 4 (2–12) pg/ml (adjusted P value = 0.045) and 123 (47–240) compared with 56 (31–133) pg/ml (adjusted P value = 0.054) respectively]. IL8 levels were elevated in obese patients following TLR2 stimulation, but not following TLR4 stimulation [68 (35–147) compared with 37 (23–63) pg/ml (adjusted P value = 0.042) and 560 (306–857) compared with 475 (226–730) pg/ml (adjusted P value = 0.729) respectively] (Figure 2).

As a marker for early activation, CD11b expression was measured at baseline in a consecutive subset of patients in both studies. In the PCI and CEA study, the flow cytometry measurements were included in the study protocol at a later time point. In both studies, obese patients revealed increased CD11b expression on $CD14^+$ monocytes, compared with non-obese patients, and these differences were significant in the PCI study (Figures 3A and 3B). In the CEA study, we also measured CD11b expression after TLR2 and TLR4

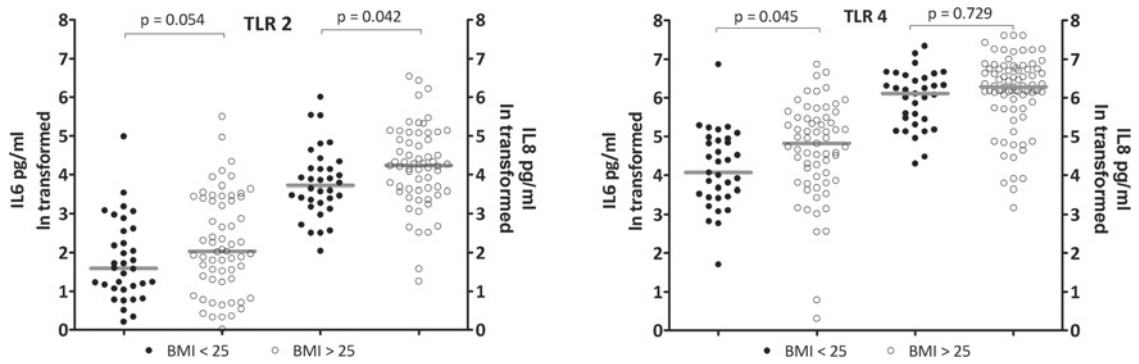

Figure 2 TLR response and obesity

IL6 and IL8 levels were determined in the CEA study following 2 h of whole blood TLR stimulation. IL-6 levels (ln-transformed) are depicted on the left *y*-axis and IL-8 levels are depicted at the right *y*-axis following TLR2 stimulation (with Pam_3CSK_4) in the left-hand panel and following TLR4 stimulation (with LPS) in the right-hand panel. (○) Obese patients; (●) non-obese patients. *P* values are adjusted for cardiovascular risk factors.

stimulation, and again, obese patients showed increased expression of the activation marker CD11b (Figures 3C and 3D). After correction for risk factors, the differences observed between obese and non-obese patients remained significant ($P = 0.009$ and $P = 0.012$ after stimulation with LPS and Pam_3CSK_4 respectively). Differences between groups were not observed after stimulation with an aspecific stimulus, PMA (Figure 3E). We did not observe differences in CD11b expression between patients with other established risk factors (Supplementary Table S3 at http://www.clinsci.org/cs/121/cs1210205add.htm). In the PCI study, we measured hsCRP. Obese patients has higher levels of hsCRP (Figure 4). Finally, TLR2 and TLR4 expression on circulating monocytes were quantified in both studies. In both studies, we did not observe differences in TLR2 and TLR4 expression between obese and non-obese patients. In the PCI study, TLR2 expression was 3.0 (2.4–3.8) and 3.3 (2.8–4.0) pg/ml and TLR4 expression was 2.4 (2.1–3.6) and 2.8 (2.5–3.5) pg/ml in the non-obese and obese patients respectively. In the CEA study, TLR2 expression was 1.5 (0.9–2.5) and 1.8 (1.0–4.3) pg/ml and TLR4 expression was 0.3 (0.2–0.5) and 0.5 (0.3–0.7) pg/ml in the non-obese and obese patients (Supplementary Table S4 at http://www.clinsci.org/cs/121/cs1210205add.htm). TLR2 and TLR4 expression also did not differ between patients without or with statins or anti-hypertensive medication (Supplementary Table S5 at http://www.clinsci.org/cs/121/cs1210205add.htm)

DISCUSSION

TLR activation results in an innate immune response and plays an important role in the pathogenesis of atherosclerosis. An association between cardiovascular risk factors and the activation status of the innate immune system, which is reflected by an altered TLR responsiveness, can be suggested. We demonstrate in two cohorts of patients, with established atherosclerosis, that obesity is a cardiovascular risk factor related with an increased TNF-α production by white blood cells following TLR activation.

Obesity and inflammation

While obesity was primarily seen as a lipid storage disease, it is nowadays recognized that obesity should be considered as a chronic inflammatory disorder [16–18]. Several studies demonstrated that obese patients have increased levels of circulating biomarkers reflecting systemic inflammation [19,34]. hsCRP is one of the most commonly studied biomarkers and is found to be elevated in obese patients [34]. Our present findings also confirmed that obese patients have increased levels of hsCRP (Figure 3). Another activation marker of the immune cells is CD11b. CD11b forms together with CD18, the Mac-1 (macrophage-1 antigen or complement receptor 3), and is regarded as a key integrin in mononuclear cell adhesion towards activated endothelium [35]. In both studies, obesity was associated with increased CD11b expression on $CD14^+$ monocytes (Figure 3). In the CEA study, CD11b expression was also measured after TLR stimulation, which resulted in significant differences between obese and non-obese patients. Stimulation with the aspecific stimulus PMA (positive control) did not show any differences between obese and non-obese patients.

Zhang et al. [36] showed that physiological levels of NEFAs, which are elevated in obese patients, are able to up-regulate CD11b expression on human monocytes and increase monocyte adherence in an adhesion assay. CD11b is an important adhesion molecule, necessary for firm adhesion of the circulating monocyte to the activated endothelium. Enhanced expression of this integrin therefore might facilitate monocyte entry in

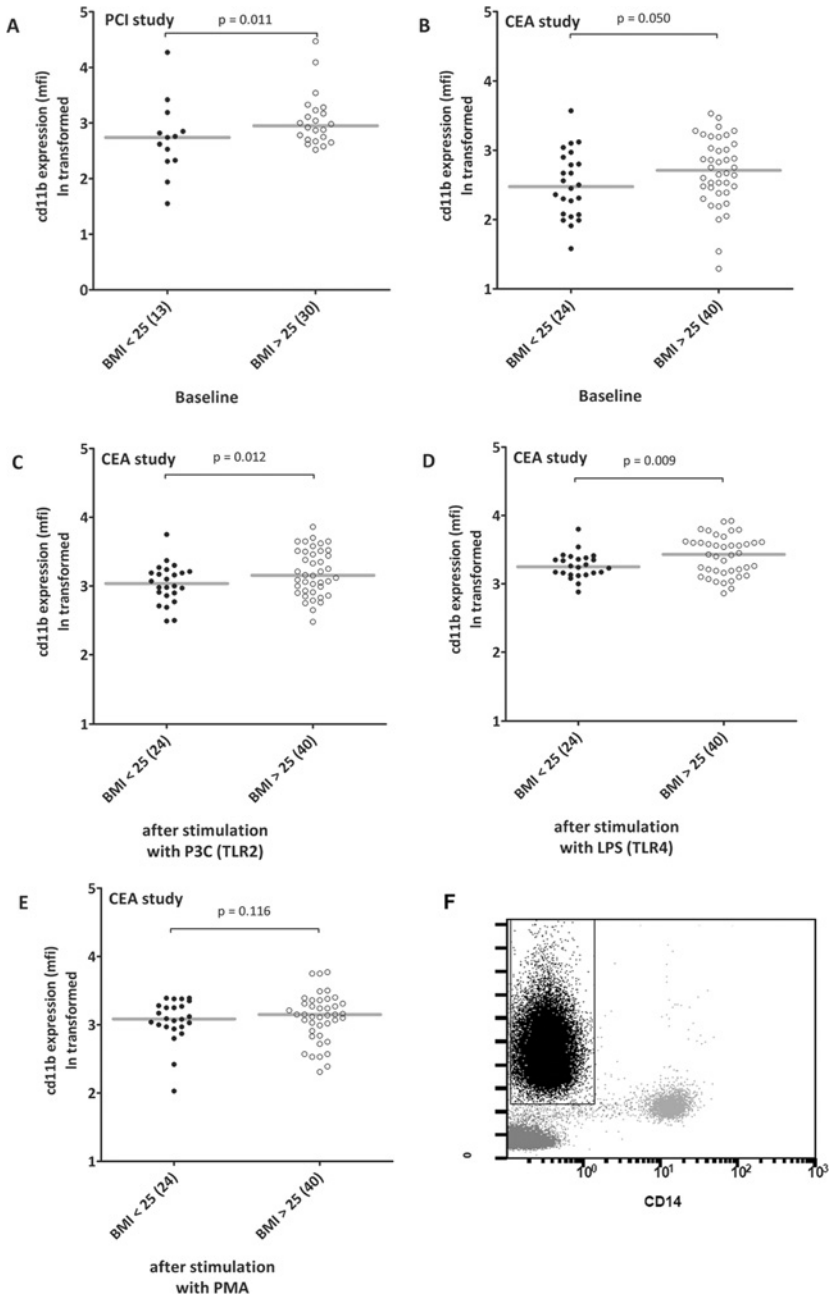

Figure 3 CD11b expression and obesity

CD11b expression (MFI, ln-transformed) on $CD14^+$ leucocytes is depicted on the *y*-axis for non-obese (●) and obese patients (○). (A) Baseline CD11b expression for the PCI study and (B) baseline CD11b expression for the CEA study. CD11b expression following TLR2 (C), TLR4 (D) and PMA (E) stimulation. (F) An example of a scatter diagram from one patient with the sideward scatter (SSC) depicted on the *y*-axis and the MFI of CD14 depicted on the *x*-axis. *P* values are adjusted for cardiovascular risk factors.

atherosclerotic lesions. This could be one possible mechanism linking obesity with atherosclerosis.

Obesity and TLR response

In the present study, obesity was the only risk factor that was associated with an increased TLR response. Obese patients have elevated levels of apoCIII (apolipoprotein CIII) and NEFAs, which are able to activate TLR2 and TLR4 respectively [37,38]. TLRs are mainly expressed on leucocytes, but adipocytes and macrophages in adipose tissue also express these receptors. Release of apoCIII or NEFAs activate TLR2 and TLR4 leading to a pro-inflammatory cytokine production. This might be an explanation of the increased levels of circulating biomarkers reflecting systemic inflammation in obese patients. Secondly, monocytes activated by these

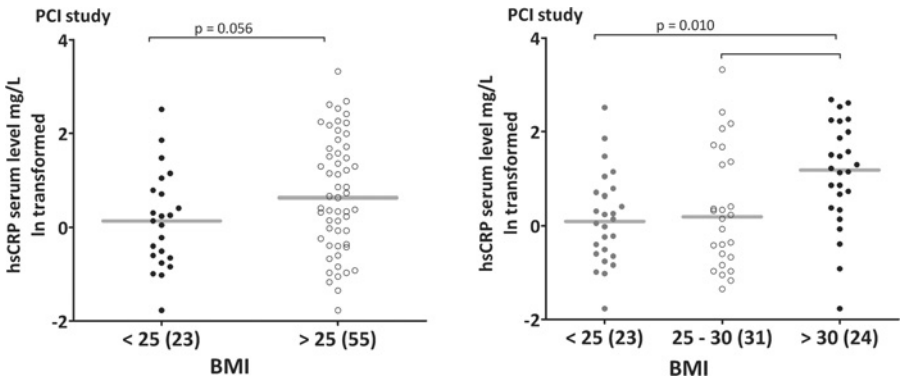

Figure 4 hsCRP levels were measured in the PCI study

hsCRP levels (ln-transformed) are depicted on the *y*-axis with the BMI of the groups depicted on the *x*-axis with non-obese compared with obese depicted in the left-hand panel and three BMI categories depicted in the right-hand panel. *P* values are adjusted for cardiovascular risk factors.

elevated levels of apoCIII or NEFAs will express more CD11b and might adhere more easily to the activated endothelium. Because of their already activated state, upon stimulation, these monocytes will produce elevated levels of pro-inflammatory cytokines, resulting in more severe atherosclerosis development.

Obese patients are also at increased risk for developing insulin resistance. In a recent review, Konner and Bruning [39] summarized the abundant evidence that links TLR activation with insulin resistance. An important role for saturated NEFAs causing insulin resistance in a TLR-dependent manner was clearly highlighted. Saturated NEFAs are able to activate IKK [inhibitor of NF-κB (nuclear factor-κB) kinase] and JNK (c-Jun N-terminal kinase) in a TLR-dependent manner. Activation of these important kinases not only results into transcription of pro-inflammatory cytokines [via NF-κB and AP-1 (activator protein-1)], but also phosphorylates IRS (insulin receptor substrate) thereby causing insulin resistance. In a large genome-wide association study, Weyrich et al. [40] demonstrated an association between the TLR4$^{D299G/T399I}$ polymorphism and increased body fat and insulin resistance in two different cohorts, supporting the role of TLR4 as a molecular link between obesity and insulin resistance.

The enhanced responsiveness we observed in obese patients could not be explained by an elevated expression of TLRs on circulating monocytes. Therefore other factors influencing TLR responsiveness might explain the increased responsiveness observed in obese patients. Schwarz et al. [41] demonstrated that saturated NEFAs, which are elevated in obese patients, are able to augment TLR-induced cytokine production in a TLR-independent manner. They showed that the saturated fatty acids lauric acid, palmitic acid or stearic acid are able to augment TLR2 and TLR4 response by stimulation of PKC (protein kinase C). Another possible explanation could be the effect of the anti-inflammatory adipokine adiponectin, levels of which are decreased in obese patients [42]. Yamaguchi et al. demonstrated that adiponectin is able to inhibit TLR2-, TLR4- and TLR9-induced NF-κB activation in murine macrophages [43]. In addition, another study showed decreased IL-6 and TNF-α production of LPS-stimulated porcine macrophages pretreated with adiponectin [44]. Although the exact mechanism is still unknown, the decreased levels of adiponectin in obese patients might explain the observed increased responsiveness in such patients.

Smoking and TLR response

In the PCI study, we observed a trend towards lower levels of TNF-α following TLR stimulation in patients who are active smokers (Table 2). When taking into account the time patients had stopped smoking, there was a trend towards higher TNF-α levels for patients who had stopped >6 months or had never smoked (Table 2). This trend was not observed in the CEA study. Chen et al. [45] showed decreased TLR2 and TLR4 responsiveness in alveolar macrophages isolated from active smokers compared with healthy controls, but failed to demonstrate decreased responsiveness of smokers PBMCs (peripheral blood mononuclear cells), following 6 h of stimulation. We only observed the decreased TLR response in the PCI study, following a longer period of stimulation (o/n incubation). This might explain the differences we observed between our two studies and the study performed by Chen et al.

The strength of our present study is that we demonstrated increased responsiveness in obese patients in two independent cohorts with established atherosclerosis of two different vascular territories. Another strength, which is also a limitation of this study, is the different stimulation times used in both studies. It shows that differences can be observed between obese and non-obese patients despite changes in stimulation duration. On the other hand, we cannot state that we reproduced the results using an identical study design. Secondly, flow cytometry measurements were not performed after

TLR stimulation in the PCI study. Finally, our present study is descriptive, and a control group of obese patients without cardiovascular disease is lacking. We can only speculate upon possible explanations for the increased responsiveness observed in obese patients. It would be of great interest to further explore this observation to see whether healthy obese patients, without clearly established atherosclerosis, have also an increased TLR responsiveness. In addition, it would be interesting to see whether more specific markers of activation, such as CD11c, are also elevated in obese patients.

Conclusions

Obese patients with established atherosclerosis show an increased whole blood TLR response and increased expression of the activation marker CD11b.

AUTHOR CONTRIBUTION

Dik Versteeg performed the PCI study. Vincent Scholtes performed the CEA study. Arjan Schoneveld provided technical supervision and set up the multiplex immunoassay system. Analyses were performed by Vincent Scholtes with help from Karlijn van Keulen and Imo Hoefer. Pieter Stella and Pieter Doevendans are interventional cardiologists and, together with Jean Paul de Vries and Frans Moll (vascular surgeons), made it possible to obtain blood prior to intervention. Gerard Pasterkamp designed the hypothesis of the study and supervised the entire study. Dominique de Kleijn, Jean-Paul de Vries and Gerard Pasterkamp revised the first versions of the manuscript. All of the authors had full access to the data and take responsibility for its integrity and the accuracy of the analysis. All authors have read and agree to the manuscript as written.

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SUPPLEMENTARY ONLINE DATA

Toll-like receptor 2 and 4 stimulation elicits an enhanced inflammatory response in human obese patients with atherosclerosis

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Table S1 Additional information on risk factors in both studies

Values are medians (IQR), or number of patients (percentage). Risk factors were scored using the following criteria: hypertension, the use of one or more antihypertensive drugs (>3 months before intervention); hypercholesterolaemia, the use of statins for at least 3 months before intervention; and diabetes, the use of antidiabetic medication (>3 months before intervention). Systolic and diastolic tension, glucose levels and cholesterol, HDL (high-density lipoprotein)-cholesterol, triacylglycerol and LDL (low-density lipoprotein)-cholesterol levels in patients with or without hypertension, diabetes or hypercholesterolemia respectively are depicted. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

	PCI study ($n = 78$)		CEA study ($n = 104$)	
Characteristic	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Hypertension (*n*)	52 (67%)	13 (57%)	83 (%)†	17 (%)†
Systolic tension (mmHg)	NA‡	NA‡	148 (134–174)	148 (130–172)
Diastolic tension (mmHg)	NA‡	NA‡	80 (71–87)	80 (68–95)
Diabetes	16 (21%)	62 (79%)	56 (20%)§	18 (22%)§
Glucose (mmol/l)	8.60 (6.55–12.88)***	5.35 (4.80–6.10)***	7.25 (5.68–9.28)*	6.10 (5.50–6.88)*
Hypercholesterolaemia	72 (92%)	6 (8%)	39‖	12‖
Cholesterol (mmol/l)	4.30 (3.70–4.80)	5.05 (4.68–6.53)	4.10 (3.40–5.10)	4.55 (4.23–5.58)
HDL-cholesterol (mmol/l)	1.12 (0.97–1.38)	1.00 (0.81–1.50)	1.15 (0.88–1.36)	1.48 (0.89–1.65)
Triacylglycerol (mmol/l)	1.51 (1.17–1.80)	0.90 (1.13–2.86)	1.5 (0.95–2.25)	1.20 (0.90–2.00)
LDL-cholesterol (mmol/l)	1.95 (1.33–2.42)**	2.92 (2.28–3.96)**	2.10 (1.73–2.70)**	3.00 (2.60–3.40)**

†Systolic and diastolic pressure values could not be obtained from four patients.
‡Systolic and diastolic pressure values could not be retrieved from patients of the PCI study.
§Glucose levels from 30 patients were missing.
‖Cholesterol, triacylglycerol, HDL-cholesterol and LDL-cholesterol values from 53 patients were missing.

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Table S2 TNF-α levels following TLR2 and TLR4 stimulation and statin, calcium channel blocker, angiotensin-converting enzyme inhibitor or angiontensin II inhibitor use

Values are medians (IQR). CBB, calcium channel blocker; ACEI, angiotensin-converting enzyme inhibitor; ATII, angiotensin II inhibitor.

(a) TLR2 stimulation

	TNF-α (pg/ml) following TLR2 stimulation			
	PCI study		CEA study	
Medication	Risk factor present	Risk factor not present	Risk factor present	Risk factor not present
Statin	12 (4–42) ($n=72$)	8 (1–23) ($n=6$)	11 (6–23) ($n=84$)	16 (8–33) ($n=20$)
CCB	12 (4–4) ($n=24$)	13 (6–41) ($n=54$)	11 (7–15) ($n=24$)	13 (7–27) ($n=80$)
ACEI	9 (1–17) ($n=19$)	13 (5–43) ($n=59$)	11 (7–18) ($n=33$)	13 (6–26) ($n=71$)
ATII	—	—	12 (7–25) ($n=27$)	13 (6–28) ($n=77$)
Statin, CCB, ACE or ATII	12 (4–40) ($n=76$)	42 ($n=2$)	12 (7–26) ($n=97$)	15 (5–18) ($n=7$)

(b) TLR4 stimulation

	TNF-α (pg/ml) following TLR4 stimulation			
	PCI study		CEA study	
Medication	Risk factor present	Risk factor not present	Risk factor present	Risk factor not present
Statin	223 (113–328) ($n=72$)	219 (196–287) ($n=6$)	181 (102–304) ($n=84$)	184 (146–291) ($n=20$)
CBB	191 (95–265) ($n=24$)	239 (128–334) ($n=54$)	164 (126–274) ($n=24$)	202 (101–320) ($n=80$)
ACEI	209 (127–342) ($n=19$)	226 (100–317) ($n=59$)	164 (99–254) ($n=33$)	204 (112–322) ($n=71$)
ATII	—	—	178 (109–322) ($n=27$)	196 (94–276) ($n=77$)
Statin, CCB, ACE or ATII	219 (118–318) ($n=76$)	297 ($n=2$)	178 (103–300) ($n=97$)	202 (149–265) ($n=7$)

Table S3 Risk factors for atherosclerosis and CD11b expression on CD14$^+$ monocytes at baseline

Values are medians (IQR), together with number of patients depicted in parentheses, for the different risk factors $^*P<0.05$; $^{(*)}P=0.05$.

	CD11b expression (MFI)			
	PCI study		CEA study	
Risk factor	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Hypertension	21 (17–67) ($n=29$)	17 (14–25) ($n=14$)	14 (10–20) ($n=51$)	13 (9–21) ($n=13$)
Diabetes	51 (15–72) ($n=4$)	18 (15–25) ($n=39$)	17 (9–23) ($n=13$)	14 (10–20) ($n=51$)
Hypercholesterolaemia	18 (14–26) ($n=37$)	26 (16–39) ($n=6$)	13 (9–20) ($n=52$)	16 (12–23) ($n=12$)
Family history of heart disease <60 years	19 (14–26) ($n=22$)	18 (14–29) ($n=21$)	16 (9–20) ($n=15$)†	13 (10–18) ($n=38$)†
BMI (BMI <25–BMI >25)	16 (10–21) ($n=13$)*	20 (16–29) ($n=33$)*	12 (8–18) ($n=24$)$^{(*)}$	15 (11–22) ($n=40$)$^{(*)}$
Smoking	19 (10) ($n=2$)	18 (15–27) ($n=41$)	12 (10–18) ($n=18$)	15 (10–21) ($n=46$)
Active smoker–past smoker <6 months	19 (10) ($n=2$)	20 (15–26) ($n=10$)	12 (10–18) ($n=18$)	13 (8–16) ($n=11$)
Past smoker >6 months–no smoking ever	17 (14–26) ($n=20$)	22 (17–22) ($n=11$)	16 (12–22) ($n=27$)	19 (7–22) ($n=8$)

†From 18 patients, no data could be obtained concerning family history of heart disease; these patients were excluded for this analysis.

Table S4 Risk factors for atherosclerosis and TLR2 and TLR4 expression on CD14$^+$ monocytes at baseline

Values are medians (IQR). $^*P < 0.05$.

(a) TLR2 expression

	TLR2 expression (MFI)			
	PCI study		CEA study	
Risk factor	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Hypertension	3.3 (2.7–4.1) ($n = 29$)	3.2 (2.4–3.7) ($n = 14$)	2.0 (1.3–4.3) ($n = 51$)	1.4 (0.9–2.7) ($n = 13$)
Diabetes	3.0 (2.4–3.8) ($n = 4$)	3.3 (2.7–4.0) ($n = 39$)	1.8 (1.0–3.5) ($n = 13$)	1.5 (0.9–2.5) ($n = 51$)
Hypercholesterolemia	3.3 (2.7–4.0) ($n = 37$)	3.3 (2.4–4.3) ($n = 6$)	1.4 (0.9–2.5) ($n = 52$)*	3.0 (1.4–7.4) ($n = 12$)*
Family history of heart disease <60 years	3.2 (2.7–3.9) ($n = 22$)	3.3 (2.6–4.0) $n = 21$)	1.5 (0.9–3.1) ($n = 15$)†	1.4 (1.0–2.8) ($n = 38$)†
BMI (BMI <25–BMI >25)	3.0 (2.4–3.8) ($n = 13$)	3.3 (2.8–4.0) ($n = 33$)	1.5 (0.9–2.5) ($n = 24$)	1.7 (0.9–3.6) ($n = 40$)
Smoking	2.33 (2.27) ($n = 2$)	3.3 (2.8–4.0) ($n = 43$)	1.1 (0.9–2.5) ($n = 18$)	1.7 (1.0–2.9) ($n = 46$)
Active smoker–past smoker <6 months	2.33 (2.27) ($n = 2$)	3.3 (2.8–4.3) ($n = 10$)	1.1 (0.9–2.5) ($n = 18$)	1.3 (0.8–1.8) ($n = 11$)
Past smoker >6 months–no smoking ever	3.3 (2.6–4.1) ($n = 22$)	3.0 (2.7–3.8) ($n = 11$)	1.7 (1.2–3.1) ($n = 27$)	2.4 (1.1–4.5) ($n = 8$)

(b) TLR4 expression

	TLR4 expression (MFI)			
	PCI study		CEA study	
Risk factor	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Hypertension	3.09 (2.41–3.85) ($n = 29$)	2.57 (2.33–2.76) ($n = 14$)	0.41 (0.21–0.84) ($n = 51$)	0.46 (0.26–0.62) ($n = 13$)
Diabetes	2.94 (1.88) ($n = 4$)	2.70 (2.42–3.56) ($n = 39$)	0.62 (0.28–0.93) ($n = 13$)	0.42 (0.23–0.61) ($n = 51$)
Hypercholesterolaemia	2.85 (2.41–3.61) ($n = 37$)	2.54 (2.24–2.76) ($n = 6$)	0.43 (0.24–0.84) ($n = 52$)	0.58 (0.35–0.84) ($n = 12$)
Family history of heart disease <60 years	2.65 (2.43–3.75) ($n = 22$)	2.75 (2.29–3.43) ($n = 21$)	0.53 (0.43–0.61) ($n = 15$)†	0.39 (0.24–0.65) ($n = 38$)†
BMI (BMI <25–BMI >25)	2.44 (2.13–3.56) ($n = 13$)	2.78 (2.51–3.50) ($n = 33$)	0.38 (0.22–0.53) ($n = 24$)	0.52 (0.27–0.76) ($n = 40$)
Smoking (yes–no)	2.27 (1.88) ($n = 2$)	2.78 (2.42–3.50) ($n = 43$)	0.37 (0.22–0.73) ($n = 18$)	0.46 (0.25–0.71) ($n = 46$)
Active smoker–past smoker <6 months	2.27 (1.88) ($n = 2$)	2.55 (2.47–2.92) ($n = 10$)	0.37 (0.22–0.73) ($n = 18$)	0.34 (0.22–0.46) ($n = 11$)
Past smoker >6 months–no smoking ever	3.27 (2.30–3.85) ($n = 22$)	2.94 (2.37–3.37) ($n = 11$)	0.51 (0.34–0.75) ($n = 27$)	0.55 (0.18–1.00) ($n = 8$)

†From 18 patients, no data could be obtained concerning family history of heart disease; these patients were excluded for this analysis.

Table S5 TLR2 and TLR4 expression on CD14$^+$ monocytes at baseline and statin, calcium channel blocker, angiotensin-converting enzyme inhibitor or angiontensin II inhibitor use

Values are medians (IQR). $^*P < 0.05$. CBB, calcium channel blocker; ACEI, angiotensin-converting enzyme inhibitor; ATII, angiotensin II inhibitor.

(a) TLR2 expression

	TLR2 expression (MFI)			
	PCI study		CEA study	
Medication	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Statin	3.3 (2.7–4.0) ($n = 39$)	3.3 (2.4–4.3) ($n = 6$)	1.4 (0.9–2.5) ($n = 53$)*	3.3 (1.3–7.5) ($n = 11$)*
CCB	3.0 (2.4–4.3) ($n = 12$)	3.3 (2.7–4.0) ($n = 33$)	1.2 (0.7–3.0) ($n = 9$)	1.6 (1.0–1.6) ($n = 55$)
ACEI	3.3 (2.9–4.0) ($n = 11$)	3.0 (2.6–4.0) ($n = 34$)	1.3 (0.8–2.8) ($n = 18$)	1.7 (1.0–2.8) ($n = 46$)
ATII	–	–	1.5 (0.9–4.4) ($n = 16$)	1.7 (1.0–2.8) ($n = 48$)
Statin, CCB, ACE or ATII	3.3 (2.7–4.0) ($n = 43$)	2.7 ($n = 2$)	1.5 (0.9–2.6) ($n = 60$)	4.5 (1.3–7.8) ($n = 4$)

(b) TLR2 expression

	TLR4 expression (MFI)			
	PCI study		CEA study	
Medication	Risk factor present	Risk factor absent	Risk factor present	Risk factor absent
Statin	2.8 (2.4–3.6) ($n = 39$)	2.5 (2.2–2.8) ($n = 6$)	0.4 (0.2–0.6) ($n = 53$)	0.6 (0.4–0.9) ($n = 11$)
CCB	2.9 (2.2–3.5) ($n = 12$)	2.7 (2.4–3.5) ($n = 33$)	0.5 (0.3–0.6) ($n = 9$)	0.4 (0.2–0.8) ($n = 55$)
ACEI	3.2 (2.1–3.8) ($n = 11$)	2.7 (2.4–3.4) ($n = 34$)	0.5 (0.3–0.6) ($n = 18$)	0.4 (0.2–0.7) ($n = 46$)
ATII	–	–	0.4 (0.2–1.2) ($n = 16$)	0.5 (0.3–0.6) ($n = 48$)
Statin, CCB, ACE or ATII	2.7 (2.4–3.5) ($n = 43$)	2.7 ($n = 2$)	0.4 (0.2–0.6) ($n = 60$)*	0.8 (0.7–0.9) ($n = 4$)*

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