Toll-like receptors (TLRs) are key pattern-recognition receptors in innate immunity, the first line of host defense against infectious agents. Collectively, TLRs recognize a variety of pathogen-associated microbial patterns. TLR-2 is essential for the recognition of bacterial lipoproteins, whereas TLR-4 is activated by lipopolysaccharide.1 There is growing evidence that TLR recognition is not restricted to exogenous microbial patterns. Putative endogenous TLR ligands, including fibronectin extra domain A fragment, heat shock proteins, and hyaluronan fragments, may be released during tissue damage and drive inflammation in the absence of infection.2

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The members of the TLR family share a cytoplasmic domain similar to a portion of the interleukin-1 receptor (IL-1R), known as the Toll/IL-1R homologous region or TIR domain. As a result, TLRs activate signaling pathways that are similar to those engaged by IL-1. The TIR domain recruits the adaptor protein myeloid differentiation primary response gene 88 (MyD88), which activates IL-1R–associated kinases. IL-1R–associated kinases in turn activate the tumor necrosis factor (TNF) receptor–associated factor 6, eliciting downstream signaling via nuclear factor-κB (NF-κB) and mitogen-activated protein kinase pathways. The MyD88-dependent pathway is shared by all TLRs, with the exception of TLR-3. TLR-4 signaling is the most complex and encompasses both the MyD88-dependent and the MyD88-independent pathways.1,3 The MyD88-independent pathway, engaged by TLR-3 and -4, relies on TIR-domain–containing adaptor protein inducing interferon-β (TRIF) to mediate interferon regulatory factor-3 and NF-κB activation.3 TLR-4 utilizes
TRIF-related adaptor molecule (TRAM) to interact with TRIF and engage the MyD88-independent pathway.3,5 Toll/IL-1R signaling is involved in the development of aortic lesions in murine atherosclerosis. Crossing of MyD88−/− mice with apolipoprotein E−/− mice has been shown to reduce the development of atherosclerotic lesions by ∼60%.6 Interruption of IL-1 signaling reduces atherosclerotic lesion formation from 40% to 67%.7,8 Similarly, targeted deletion of TLR-4 or TLR-2 in apolipoprotein E−/− mice resulted in a 55% reduction of atherosclerotic lesion development.9–12 Local delivery of lipopolysaccharide affects arterial remodeling after arterial injury.13 Exposure to synthetic TLR-2 ligands or live bacteria induces a significant increase in atherosclerosis10,12 and neointima formation.14 TLR-2 expression is increased in endothelial cells at regions of susceptibility of atherosclerosis, and it is associated with monocyte recruitment.15 Notwithstanding the evidence of the role of TLR signaling in experimental models of atherosclerosis, the functional role of TLRs in human atherosclerosis is yet to be proved. Increased expression of TLR-1, -2, and -4 is found in macrophages and resident vascular cells in human atherosclerosis.16 Two cosegregating TLR-4 gene point mutations (Asp299Gly and Thr399Ile) related to a blunted response to inhaled lipopolysaccharide have been investigated with contradictory results. For instance, the TLR-4Asp299Gly polymorphism has been associated with a reduced intima-media carotid thickness.17 However, in the Carotid Artery Progression Study, this polymorphism was not linked to intima-media thickness or plaque progression over a 3-year follow-up.18 Both polymorphisms have been found at a higher frequency in a cohort of patients with myocardial infarction.19 The TLR-2 polymorphism Arg753Gln, shown to confer increased expression of TLR-1, -2, and -4 is found in experimental models of atherosclerosis, the functional role of TLRs in human atherosclerosis is yet to be proved. Increased expression of TLR-1, -2, and -4 is found in macrophages and resident vascular cells in human atherosclerosis.16 Two cosegregating TLR-4 gene point mutations (Asp299Gly and Thr399Ile) related to a blunted response to inhaled lipopolysaccharide have been investigated with contradictory results. For instance, the TLR-4Asp299Gly polymorphism has been associated with a reduced intima-media carotid thickness.17 However, in the Carotid Artery Progression Study, this polymorphism was not linked to intima-media thickness or plaque progression over a 3-year follow-up.18 Both polymorphisms have been found at a higher frequency in a cohort of patients with myocardial infarction.19 The TLR-2 polymorphism Arg753Gln, shown to confer susceptibility to mycobacteria infection, was associated with restenosis after coronary angioplasty.20 Finally, most studies on TLR-2 and TLR-4 in human atherosclerotic diseases have been performed with the use of peripheral blood monocytes. Patients with acute coronary syndromes but not stable angina have an increased expression of TLR-2 and -4 on circulating monocytes.21,22 Such increase in expression does not always result in enhanced TLR signaling.23,24 Therefore, there is currently little information on whether TLR signaling is protective or detrimental at lesion sites in human atherosclerosis. We have previously shown the feasibility of isolating viable cells from human carotid atherosclerotic lesions from patients undergoing surgical endarterectomy. This system models the cellular interactions taking place in vivo and permits the direct investigation of signaling pathways. This complex mixture contains macrophages, lymphocytes, and smooth muscle cells and displays in culture a spontaneous production of proinflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs).25 Such dysregulated production depends on the NF-κB pathway and could be significantly prevented by blocking IkB kinase-β activity.25 Given the variety of receptors signaling through NF-κB, the upstream determinants of NF-κB activation in human atherosclerosis are still unknown.

Genetic deletions of TLR-2, TLR-4, IL-1, and MyD88 signaling, all leading to NF-κB activation, have an equivalent effect on murine atherosclerosis.6,9,10 Here we show that, in human atherosclerosis, TLR-2 and MyD88 play a predomi- nant role in NF-κB activation and in the production of inflammatory mediators and even matrix-degrading enzymes.

**Methods**

**Patient Population and Sample Collection**

Carotid endarterectomies from 58 patients undergoing revascularization procedures for symptomatic carotid disease were obtained at Charing Cross Hospital, London. The protocol was approved by the Research Ethics Committee. All patients gave written informed consent, according to the Human Tissue Act 2004 (UK).

**Ex Vivo Culture of Cells Isolated From Human Atherosclerotic Plaques**

Diseased intimal arterial segments were dissected from carotid endarterectomy specimens and single cell suspensions obtained by enzymatic digestion, as published previously.23 The viable cell number obtained was 3 ± 1.9 × 10⁶ (mean ± SEM). Phenotyping by flow cytometry (Methods in the online-only Data Supplement) confirmed our previous observation25 that macrophages are the most abundant cells in the mixture, being approximately double the proportions of smooth muscle cells and lymphocytes (Figure Ia in the online-only Data Supplement). Apoptosis was assayed with the use of propidium iodide (PI) and annexin V (Methods in the online-only Data Supplement; Figure Ib in the online-only Data Supplement).

**Blockers**

Freshly isolated atheroma cells were cultured at 10⁶ cells per milliliter in RPMI containing 10% fetal bovine serum (Biosera, UK) either alone or in the presence of anti-human TLR-2 (clone T2.2, eBioscience) and TLR-4 (clone HTA125, Abcam) antibodies or isotype-control antibody (eBioscience) at a concentration of 25 μg·mL⁻¹ (based on dose-ranging experiments; data not shown). The antibodies used were selected for their efficacy in inhibiting TLR-4 and TLR-2 signaling in human umbilical vein endothelial cells26 and primary macrophages27 (Methods in the online-only Data Supplement; Figures II and III in the online-only Data Supplement). IL-1R antagonist (IL-1Ra) (R&D Systems) was used at a concentration of 10 μg·mL⁻¹. Supernatants were removed after 24, 48, 72, and 96 hours and stored at −80°C for single-batch analysis of cytokine and MMP production via Luminex and zymography (Methods in the online-only Data Supplement). Viability was monitored with the use of 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma, UK).28 NF-κB activation on blockers was assessed via NF-κB luciferase reporter assay29 (Methods in the online-only Data Supplement). Each condition was tested in triplicate culture, and each triplicate was analyzed separately.

**Adenoviral Vectors**

E1/E3-deleted recombinant replication-deficient adenoviral vectors (serotype Ad5) encoding green fluorescent protein (GFP), either alone (control Ad) or with a dominant-negative (DN) form of MyD88 (AdMyD88DN) or TRAM (AdTRAMDN), were generated as described.27,29,30 Cells were plated at 10⁶ cells per milliliter in RPMI and either left uninfected or infected in serum-free conditions for 2 hours with indicated adenoviruses at multiplicity of infection 200:1, which results in >90% infection efficiency, as shown previously.25 After 48 and 72 hours, supernatants were removed and stored at −80°C. Cellular viability and NF-κB activity were assessed. Successful infection was monitored via the GFP tag and immunoblotting (Methods in the online-only Data Supplement).

**Statistical Analysis**

Data were analyzed with Stata (version 10, StataCorp LP, Tex). We used nonparametric methods to account for the nonnormality of distributions. Rank ANCOVA was used to assess the effect of treatment with the indicated blockers, adjusted for time (in culture) as a covariate. Alternatively, the Wilcoxon rank sum test was used to
Figure 1. Successful adenoviral gene transfer targeting MyD88 in cells isolated from human atherosclerotic tissue. Adenoviral infection was monitored in each experiment via a GFP tag contained in adenoviruses. One representative experiment is shown (total n=6); bright light field (A) and direct immunofluorescence (B) are shown; magnification ×20. C, Expression of the transgene was monitored by immunoblotting for MyD88 and α-tubulin. Representative blots are shown (total n=3). Successful overexpression of the transgenes compared with endogenous levels was demonstrated in all occasions. Ab indicates antibody. D, MyD88DN overexpression did not affect cell viability. Cellular viability was assessed by MTT. Values are percentage relative to uninfected cells (mean±SEM; n=10). There was no significant difference in viability between uninfected and adenovirus-infected cells (P>0.05, rank ANCOVA). E, MyD88 drives NF-κB activation in human atheroma cell cultures. NF-κB activation after 48 hours in culture with indicated adenoviruses was monitored by NF-κB luciferase reporter assay. Values are percentage of luciferase activity relative to uninfected cells (mean±SEM; n=3). No significant difference was observed between uninfected cells and control adenovirus–infected cells (P>0.05). Significant inhibition of NF-κB was achieved with AdMyd88DN versus control Ad–infected cells (P<0.05, Wilcoxon rank sum test).

Figure 2. MyD88 is involved in cytokine, chemokine, and MMP production in human atheroma cell cultures. Values are percentage relative to uninfected cells (mean±SEM; n=10). No significant difference was observed between uninfected cells and control Ad–infected cells (P>0.05). AdMyD88DN caused statistically significant decreases in the production of MCP-1 (***P=0.000; A); IL-8 (**P=0.006; B); IL-6 (**P=0.002; C); MMP-1 (**P=0.002; D), and MMP-3 (***P=0.000; E) vs control Ad–infected cells (rank ANCOVA).

Results

MyD88 Signaling Plays a Key Role in Driving NF-κB Activation and the Production of Proinflammatory Mediators and MMPs in Human Atheroma Cell Cultures

To evaluate the overall effect of blocking the TLR/IL-1 pathway, we first overexpressed a dominant-negative form of MyD88 in atheroma cell cultures through adenoviral gene transfer (AdMyD88DN). Successful overexpression of the transgenes, compared with endogenous levels, was demonstrated by the expression of adenovirus-contained GFP (Figure 1A and 1B) and immunoblotting (Figure 1C). There was no significant difference in viability between uninfected and adenovirus-infected cells (Figure 1D). Similarly, there was no difference in PI/annexin V staining between infected and uninfected cells in the overall cell population (<15% annexin V– and 5% PI-positive cells in all conditions) or in different cell types (data not shown).

Using an NF-κB reporter gene under a luciferase promoter, we found that NF-κB activation was inhibited in AdMyD88DN–infected cells versus control-Ad–infected cells (P<0.05; Figure 1E). AdMyD88DN caused statistically significant decreases in the production of monocyte chemotactic protein-1 (MCP-1/CCL2; P=0.000), IL-8/CXCL8 (P=0.006), and IL-6 (P=0.002; Figure 2A to 2C) but not TNF-α (Table I in the online-only Data Supplement). Production of MMP-1 and MMP-3 was also significantly reduced (Figure 2D and 2E; Table I in the online-only Data Supplement). These results indicate that MyD88–dependent signaling is involved in inflammation and matrix degradation in human atherosclerotic plaques.

IL-1 Blockade Has Only a Partial Effect on NF-κB Activation and Cytokine Production

Because MyD88 is shared by TLR and IL-1 signaling, we explored the effect of IL-1 blockade with a natural inhibitor of IL-1 activity, IL-1Ra. Treatment with IL-1Ra achieved reduction of NF-κB activation (22±5%; P=0.05; n=3; data not shown) and IL-6 (P=0.009; 45±9% inhibition at 72 hours; Figure 3A). However, there was no effect on MMP-3 (Figure 3B), MCP-1/CCL2, or other MyD88-dependent mediators in atheroma cell cultures (data not shown).

TLR-2 and TLR-4 Drive NF-κB Activation

Because MyD88 but not IL-1 blockade resulted in reduction of several proatherogenic mediators, we assessed the functional relevance of alternative MyD88 activators, such as...
TLR-2 and TLR-4, using anti-TLR-2 and -4 blocking antibodies. To assess whether NF-κB activation in human atheroma cell culture is dependent on TLR-2 and TLR-4, we utilized a NF-κB luciferase reporter assay. TLR-2 and TLR-4 blocking antibodies caused 60/11006 6% and 38/11006 5% reduction, respectively, in NF-κB activation compared with the isotype control (P<0.05; Figure 4A). Such reduction of NF-κB activity was not associated with changes in cell viability, as assessed by MTT assay (Figure 4B) or PI/annexin V staining (<15% annexin V– and 5% PI-positive cells in the overall cell population in all conditions).

**Figure 3.** Effect of IL-1 blockade on IL-6 and MMP-3 production. Data are shown as mean±SEM (n=15). IL-1Ra significantly reduced IL-6 (P<0.009; A) compared with untreated cells but not MMP-3 production (P>0.05, rank ANCOVA; B).

TLR-2 and TLR-4 blockade significantly reduces cytokine and chemokine production in advanced human carotid atherosclerotic lesions. Culture of atheroma cells in the presence of anti-TLR-2 antibodies caused significant reduction in MCP-1/CCL2 (P<0.000; Figure 5A), IL-8/CXCL8 (P<0.009; Figure 5B), IL-6 (P<0.000; Figure 5C), MMP-1 (P<0.000; Figure 5D), MMP-2 (P<0.004; Figure 5E), MMP-3 (P<0.000; Figure 5F), MMP-9 (P<0.006; Figure 5G), and MMP-9 (P<0.006; Figure 5H) production vs isotype control-treated cells. The TLR-4 blocking antibody (Ab) reduced MMP-3 (P<0.000; rank ANCOVA).

**Figure 4.** TLR-2 and -4 blockade reduces NF-κB activity in human atherosclerosis. A, NF-κB activation after 48 hours in culture with indicated blockers was monitored via a NF-κB luciferase reporter assay. Values are percentage relative to untreated cells (mean±SEM; n=6). Statistically significant inhibition of NF-κB was achieved with TLR-2 and TLR-4 blockers vs isotype control (n=6; *P<0.05, Wilcoxon rank sum test). Ab indicates antibody. B, Cellular viability was assessed by MTT. Values are percentage relative to untreated cells (mean±SEM; n=6). TLR-2 and TLR-4 blockade does not affect cell viability (P>0.05 vs isotype control–treated cells, rank ANCOVA).

**Figure 5.** TLR-2 blockade significantly reduces cytokine, chemokine, and MMP production in human atheroma cell cultures. Data are shown as mean±SEM (n=6). No significant difference was observed between untreated cells and isotype control–treated cells (P>0.05; data not shown). TLR-2 blocking antibodies significantly reduced MCP-1 (P<0.000; Figure 5A), IL-8 (P<0.009; Figure 5B), IL-6 (P<0.000; Figure 5C), MMP-1 (P<0.000; Figure 5D), MMP-2 (P<0.004; Figure 5E), MMP-3 (P<0.000; Figure 5F), MMP-9 (P<0.006; Figure 5G), and MMP-9 (P<0.006; Figure 5H) production vs isotype control–treated cells. The TLR-4 blocking antibody (Ab) reduced MMP-3 (P<0.000; rank ANCOVA).

**TLR-2 Blockade Significantly Reduces Cytokine and Chemokine Production**

We sought to evaluate whether TLR-2 and TLR-4 blockade would directly modulate the production of cytokines and chemokines in advanced human carotid atherosclerotic lesions. Culture of atheroma cells in the presence of anti-TLR-2 antibodies caused significant reduction in MCP-1/CCL2 (P<0.000), IL-8/CXCL8 (P<0.009), and IL-6 production (P<0.000; Figure 5A, 5B, and 5D) compared with the isotype control, whereas TNF-α production was not affected (Figure 5C). Percent inhibitions at each time point are shown in Table II in the online-only Data Supplement. In contrast, the anti-TLR-4 antibody did not significantly affect the production of cytokines and chemokines (Figure 5A through 5D).
TLR-2 Blockade Significantly Reduces MMP Production in Human Atheroma Cell Cultures

The effect of TLR-2 and TLR-4 inhibition on the production of MMPs, a key step toward plaque vulnerability to rupture in humans, is unknown. Hence, we evaluated the outcome of exposing atheroma cell cultures to TLR-2 and -4 neutralizing antibodies and quantified the production of MMP-1, -2, -3, and -9. We found that in the presence of anti-TLR-2 antibodies, production of MMP-1, -2, -3, and -9 was significantly reduced ($P<0.000$, $P=0.004$, $P=0.000$, and $P=0.006$, respectively; Figure 6E to 6H). Reduction of MMP levels was confirmed by zymography (Figure IV in the online-only Data Supplement). The effect of TLR-2 blockade was consistent across a larger donor group ($n=16$; Figure 6). Tissue inhibitor of matrix metalloproteinases-1 and extracellular matrix metalloproteinase inducer production was unaffected (Table II in the online-only Data Supplement). Anti-TLR-4 antibodies had no statistically significant effect on MMP-1, -2, and -9 (Figure 5E through 5H) but reduced MMP-3 production ($P=0.000$; Figure 5G).

Effect of TRAM Blockade on MMP Production

The lack of effect with TLR-4 neutralizing antibodies in the atheroma cell culture prompted us to use an alternative method of TLR-4 blockade, a dominant-negative form of the adaptor TRAM (TRAMDN), which is used by TLR-4.3 Overexpression of TRAMDN (Figure V as through Vc in the online-only Data Supplement) was not associated with changes in cell viability between uninfected and adenovirus-infected cells (Figure Vd in the online-only Data Supplement). TRAMDN significantly reduced NF-kB activity (Figure Ve in the online-only Data Supplement). TRAMDN significantly reduced MMP-1 ($P=0.009$) and MMP-3 ($P=0.000$; Figure 7D and 7E) but did not achieve statistically significant inhibition of the production of the other mediators (Figure 7A through 7C and 7F; Table I in the online-only Data Supplement).

Discussion

The development of an atherosclerotic plaque is characterized by the recruitment of blood-borne mononuclear cells, such as monocytes and lymphocytes, into the vessel wall and the production of proinflammatory mediators. Inflammation is thought to contribute to acute cardiovascular events by promoting the production of MMPs, which in turn weaken the mechanical strength of collagen, a main structural component of the fibrous cap.31 Expression of these molecules is normally tightly regulated. However, during development of atherosclerosis, their expression is upregulated for extended periods of time, promoting inflammation and tissue destruction.

Although targeting of a myriad of inflammatory mediators and their receptors in mice prevents the development of atherosclerotic lesions,31 translation in humans has not yet been achieved. This is potentially due to differences in lipid metabolism and inflammation between mice and humans32 and a lack of experimental tools to test efficacy in human atherosclerotic lesions in the preclinical phase. We developed a short-term cell culture system whereby cells contained in human carotid endarterectomies can be isolated alive and used for functional analyses. We demonstrated that cells isolated from human atherosclerotic lesions are characterized in culture by a dysregulated production of cytokines, which is NF-kB dependent.23 In the present study, we dissected upstream signaling components with the potential to drive NF-kB activation.

TLRs are key inducers of NF-kB signaling1 and share the signaling adaptor MyD88 with IL-1R. Previous studies in mice have shown that genetic deletion of MyD88 or TLR-2
prevents atherosclerosis development and macrophage infiltration.6,9,10 Interestingly, the decrease in lesion size is associated with a reduction of serum cholesterol levels.6,9,10 In our study, we observed that interruption of TLR-2 and MyD88 signaling in cells isolated from human atherosclerotic lesions had a direct effect on NF-κB activation, as well as cytokine and chemokine production. Both MyD88 and TLR-2 blockade did not affect TNF-α levels. In our previous studies, TNF-α production by atheroma cell culture was dependent on the function of the NF-κB pathway.25 The different levels of TLR dependence observed with the various mediators analyzed could be explained by a redundancy of signaling mechanisms in the complex human atheroma microenvironment. The lack of effect on TNF-α could be explained alternatively by the kinetics displayed by TNF-α release in our cell culture system, whereby TNF-α peaks at 24 hours, and subsequently its production fades away rapidly, and its levels become undetectable. The kinetics of other cytokines and chemokines were characterized by a steady increase over the 96-hour period in culture. Indeed, the effectiveness of TLR-2 and MyD88 inhibition on the production of these proinflammatory mediators also increased over time.

The current consensus view of the mechanisms leading to plaque rupture has assigned a central role to MMP-dependent collagen degradation. Increased expression of MMP-1 (collagenase), -3 (stromelysin), and -9 (gelatinase) has been observed in the shoulder of human carotid plaques in association with macrophages and smooth muscle cells.33 Levels of gelatinases, such as MMP-9, are predictors of adverse events in coronary artery disease and carotid artery disease.33 Polymorphisms of haplotypes leading to an increase in MMP-1 and -3 activity increase the risk of myocardial infarction and stroke.34 The potential involvement of TLR signaling in the regulation of MMP production has not yet been proven. Herein, we show that TLR-2 and MyD88 blockade not only blocked proinflammatory mediators, but it was also effective in reducing production of MMPs, suggesting that TLR signaling influences plaque vulnerability to rupture. Stimulation of macrophages and smooth muscle cells by proinflammatory cytokines, such as IL-1, leads to MMP expression.35 Because of the nature of the model system utilized, we are unable to discriminate whether the reduction of MMP release in the atheroma cell culture is attributable to TLR signaling blockade or secondary to cytokine inhibition. However, our finding that IL-1Ra did not affect MMP release indicates that TLR signaling plays a dominant role in extracellular matrix catabolism in the human atherosclerotic plaque.

IL-1 is a prototypic inflammatory cytokine that exists in 2 forms, IL-1α and IL-1β, both of which exert similar biological functions mediated through the IL-1R. IL-1Ra is a naturally occurring cytokine that binds to IL-1R but does not elicit a biological response.36 A variety of studies have shown reduction of atherosclerotic lesion formation in hyperlipidemic mice via overexpression of IL-1Ra or IL-1 deletion.7,8 Surprisingly, in our study IL-1 blockade by IL-1Ra reduced IL-6 release in atheroma cell cultures but not chemokine or MMP release. Hence, MyD88-dependent signaling in human atherosclerosis appears to be predominantly dependent on TLR rather than IL-1R signaling.

TLR-4 is unique among TLRs in being able to activate both the MyD88-dependent and the MyD88-independent pathways, leading to NF-κB and interferon regulatory factor-3 signaling.1,3,5,37 TLR-4 genetic deletion in mice was associated with a reduction of atherosclerotic lesions, macrophage recruitment, and cholesterol levels.3 In our study, blockade of TLR-4 signaling in human plaques, either via neutralizing antibodies or via overexpression of a dominant-negative form of the downstream TLR-4 signaling adaptor TRAM, reduced endogenous NF-κB activation in atheroma cells. This degree of inhibition of NF-κB activation did not achieve a significant downregulation of the NF-κB-dependent cytokines, such as TNF-α, IL-6, IL-8/CXCL8, and MCP-1/CCL2. We attempted to measure interferon regulatory factor-3–dependent genes, such as interferon-β, CXCL10, and CXCL11, but these mediators were below detection limits (data not shown). This could be either due to their low abundance or due to the fact that they are not part of the dysregulated cytokine production we sought to study. Therefore, we focused on NF-κB–dependent responses, which we characterized previously.25

The role of TRAM not only is limited to interferon regulatory factor-3 signaling but is required for late NF-κB activation and NF-κB–dependent gene production (eg, IL-6).1,3,5,37 Blockade of TRAM does not affect IL-6 production in the atheroma cell culture. This finding indicates a prevalent role of MyD88 in NF-κB–dependent proinflammatory responses in human atherosclerosis. Nevertheless, we uncovered a role for TLR-4/TRAM-dependent signaling in MMP production, suggesting that the MyD88-independent pathway may play a role in mechanisms related to matrix degradation and plaque vulnerability. Because TLR-4 can also signal through MyD88, we are unable to draw definite conclusions about the role of TLR-4 signaling. However, given the significant impact of TLR-2 blockade, a possible interpretation of our data is that TLR-2 might account for most of the MyD88-dependent responses.

This ex vivo model system of short-term culture of atheroma cells is not without potential limitations. The atheroma cell culture studies production of mediators by mixed cell populations.29 Further separation of different cell types is avoided to minimize cell manipulation and activation and is also limited by low cell numbers. As a result, we were not able to pinpoint the specific cell type affected by TLR blockade. Mullick et al10 have shown that, in contrast to whole-body deficiency of TLR-2, bone marrow transfer from TLR-2–/– to low-density lipoprotein receptor–/– mice was effective in preventing exogenous TLR-2 ligand–induced disease amplification but not baseline atherosclerotic lesion formation. This finding points toward a key role for TLR-2 expression in vascular cells.15 Of relevance, only endothelial cells, but not myeloid cells, express TLR-2 in murine lesions.15 However, in human lesions, TLR-2 expression was detected in macrophages, endothelial cells, and smooth muscle cells.16 Hence, TLR-2 blockade in human atherosclerosis may affect all cell types in the lesions. Differences in expression in TLR-2 could result either from differences
between early versus late disease stage or a difference between murine and human atherosclerosis. Moreover, the technique used to isolate and culture cells from atherosclerotic lesions, based on the well-established culture of synovial cells from the joints of patients with rheumatoid arthritis, has the potential for cell culture– and isolation-induced artifacts (eg, some degree of cell activation). However, no such cytokine production can be observed in cells cultured from atherosclerosis-free human vascular tissues, reflecting either a different cellular composition of these specimens or activation state of the cells during disease. Finally, we acknowledge the possibility that the atheroma cell culture system does not fully represent TLR-4 signaling in vivo. For instance, lipopolysaccharide contamination could lead to TLR-4 hyporesponsiveness. We addressed this concern by excluding lipopolysaccharide contamination via the sensitive Limulus amebocyte assay (Methods in the online-only Data Supplement) in supernatants and in enzymatic mixtures. An alternative explanation is the subversion of the extracellular matrix components during cell isolation. TLR-4 signaling is inhibited by intact extracellular matrix components, whereas extracellular matrix fragments might activate signaling. In support of this concept, TLR responses are attenuated during atherosclerosis progression, and fibroprotectin extra domain A fragment might have a role in TLR signaling tolerance. However, it is worth noting that, although matrix components may be present in the atheroma cell culture, they could also affect TLR-2 signaling.

We conclude that TLR-2/MyD88-dependent signaling is a key pathway in maintaining the proinflammatory and pro-catabolic phenotype of cells isolated from human atherosclerotic lesions. Our study highlights differences between human and mouse atherosclerosis that might guide therapeutic applications of TLR blockade in cardiovascular disease. Given the diversity of bacterial signatures and potential endogenous ligands detected in human atherosclerotic lesions, TLR-2 blockade may be a “broad” therapeutic strategy for atherosclerosis and its complications.

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Disclosures
None.

References
Inflammation and matrix degradation are the hallmarks of high-risk atherosclerosis that leads to myocardial infarction and stroke. Many previous studies have identified various pathways leading to atherosclerotic lesions in mice; however, this knowledge has thus far failed to translate to effective therapeutic strategies. Toll-like receptors (TLRs), key players in innate immunity, are upregulated in atherosclerotic lesions, but their functional role in human atherosclerosis is unknown. We previously showed that cytokine, chemokine, and matrix metalloproteinase production, in an in vitro model of human atherosclerosis, is dependent on nuclear factor-H9260, a key transcription factor downstream of TLR signaling. Here we have explored the effects of blocking TLR-2, TLR-4, and myeloid differentiation primary response gene 88, a signaling adaptor shared by most TLRs and interleukin-1 receptor. Blockade of TLR-2 markedly reduced the production of proinflammatory mediators and matrix metalloproteases. Similar effects were observed with myeloid differentiation primary response gene 88 inhibition, indicating that production of proinflammatory cytokines, chemokines, and matrix metalloproteinases in human atherosclerotic lesions is myeloid differentiation primary response gene 88 dependent. Our data show for the first time that in human atherosclerosis, TLR-2 signaling through myeloid differentiation primary response gene 88 is a rate-limiting step for both inflammation and matrix degradation. This study, by identifying a specific inflammatory pathway that may mediate the instability of cardiovascular lesions, forms the basis for the development of therapeutic agents targeting TLR-2 in cardiovascular disease.
Toll-Like Receptor-2 Mediates Inflammation and Matrix Degradation in Human Atherosclerosis

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Supplemental Methods

Flow cytometry

Cell were phenotyped using FITC-conjugated anti-human CD3 (Pharmingen, UK) and anti-human CD68 (Dako, Denmark), anti-human smooth muscle cell α-actin (Dako, Denmark) with goat anti-mouse IgG FITC (Pharmingen, UK), or Isotype controls, and analyzed by FACScan (Becton Dickinson) and Flow-Jo Software (TreeStar, USA).

Apoptosis was assayed using propidium iodide (PI) and annexin V (R&D Systems, UK), and analyzed by FACScan (Becton Dickinson) with Flow-Jo Software. Different populations of cells were identified via forward and side scatter analysis. PI+ cells were in all preparations below 5% (data not shown). All cell types underwent some degree of apoptosis, as assessed by percentage of Annexin V+ cells, during the culture period (Supplementary Figure 1b). However, lymphocytes underwent apoptosis to a greater extent as compared to macrophages and smooth muscle cells (P=.0001; Wilcoxon Rank Sum Test; Supplementary Figure 1b). This phenomenon is likely to be due to the culture conditions, as in this study the medium did not contain human serum or IL-2 or other added growth factors which would facilitate the survival of human lymphocytes.

TLR antibody testing in human primary cells

A variety of commercially available TLR-2 and -4 neutralizing antibodies were tested in primary human umbilical vein endothelial cells (HUVECs), and human monocyte-derived macrophages before use in the atheroma cell culture. HUVECs and peripheral blood monocytes were isolated and cultured as previously described \cite{26,27}. Macrophages were
derived from monocytes after differentiation for 2 to 3 days with 100 ng/mL macrophage colony-stimulating factor (M-CSF; R&D Systems). Cells were plated in 96- and 48-well tissue-culture plates (Falcon, Oxford, United Kingdom) and were incubated with neutralizing anti-TLR2 or anti-TLR4 antibodies or Isotype control for 30 minutes prior to addition of Ultrapure Escherichia coli LPS, Pam3CSK4 (Synthetic triacylated lipoprotein; TLR1/2 Agonist; Endotoxin free) or FSL-1 (Synthetic diacylated lipoprotein; TLR2/6 Agonist; Endotoxin-free) from Invivogen (CA, USA). Supernatants were collected at 24 hours for analysis of IL-6 by ELISA. Alternatively, cells were infected with an adenovirus containing an NFκB luciferase reporter gene (AdNFκBluc). Cells were then incubated with neutralizing anti-TLR2 or anti-TLR4 antibodies for 30 minutes prior to addition of the above stimuli. Luciferase activity was analyzed after 24 hours from stimulation.

**Limulus amebocyte assay**

The endotoxin content in all reagents and cell culture supernatants was measured with the Limulus amebocyte assay (BioWhittaker, Wokingham, United Kingdom) according to the manufacturer's instruction. LPS content in all media reagents and supernatants was always below detection limit (<10 pg/mL).

**Analysis of cytokine and MMP production**

IL6, TNFα, MCP-1/CCL2, IL-8/CXCL8, MMP-1, -2, -3, -9 were quantified by Luminex TM 100 using Fluorokine Multianalyte kits by R&D System. Alternatively, when indicated, ELISAs were performed to measure production of IL-6 (BD Pharmingen) using paired antibodies as described previously 25. Tissue inhibitor of matrix metalloproteinases -1 (TIMP-1) was detected by Human Biotrak Assay (Amersham). Extracellular matrix
metalloproteinase inducer (EMMPRIN) was detected using Human Total EMMPRIN Duoset Kit (R&D Systems). Optical density was read on a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromic) and analyzed using Ascent software V2.6 (Thermo Labsystems).

**NFκB luciferase reporter assay**

Cells were infected with an adenovirus containing an NFκB luciferase reporter gene (AdNFκBluc) constructed from a pNFκB-Reporter vector (BD Clontech, Palo Alto, CA) and kindly provided by Dr Paul McCray (University of Iowa). Cells were then cultured in the presence of TLR blocking antibodies for 48 hours. Alternatively, cells were infected with the indicated adenoviruses, and cultured for 48 hours. Luciferase activity was assessed on cell lysates, as previously published²⁹.

**Zymography**

Atheroma cell supernatants were collected at 96 hours after culture in the presence of neutralizing antibodies for TLR-4 and TLR-2 or Isotype control. Protein levels were quantified by Bradford reagent (Sigma). Protein content was assessed by Bradford assay. Equal amounts of protein were loaded per lane in a 0.1% casein 12% acrylamide (for MMP-3) or 0.1% gelatin 10% acrylamide (for MMP-2 and -9) Novex Zymogram gels (Invitrogen). Gels were then washed in Renaturing buffer (Invitrogen) and subsequently incubated in Developing Buffer (Invitrogen) at 37° for 24 hours. Finally, gels were stained with Comassie Blue in order to visualize the zymographic pattern.
**Immuno-Blotting**

Whole atheroma cells were plated at $1 \times 10^6$ cells/ml in 6-well tissue culture plates (Falcon) and were either uninfected or infected with relevant adenovirus. Whole-cell lysates were prepared, as published\textsuperscript{27}. Protein content was assessed by Bradford assay. Equal amounts of proteins were resolved on 4-12% Nupage Novex Bis-Tris Precast gels (Invitrogen), followed by electrotransfer onto polyvinyl difluoride membrane (Millipore, UK). Overexpression of the transgenes was assessed via anti-MyD88 (Abcam), anti-TRAM (generated in house as published\textsuperscript{30}) and $\alpha$-tubulin (Sigma) antibodies and horseradish peroxidase-conjugated donkey anti-rabbit antibody (DakoCytomation), and visualized by enhanced chemiluminescence reagents (Amersham).
Supplemental Table 1: Percentage inhibitions with MyD88 and TRAM targeting *versus* Control Adenovirus

<table>
<thead>
<tr>
<th></th>
<th>TNFα</th>
<th>MCP-1</th>
<th>IL-8</th>
<th>IL-6</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-9</th>
<th>TIMP-1</th>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>48h mean</td>
<td>3.35</td>
<td>43.78</td>
<td>48.31</td>
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<tr>
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Supplemental Table 2. Percentage inhibition with TLR-2 neutralizing Antibody versus Isotype Control Antibody

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<th>Time</th>
<th>TNFα</th>
<th>MCP-1</th>
<th>IL-8</th>
<th>IL-6</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-9</th>
<th>TIMP-1</th>
<th>EMMPRIN</th>
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<td>3.67</td>
<td>2.24</td>
</tr>
</tbody>
</table>
Supplementary Figure 1

(a) 

(b)
Supplementary Figure 2

a) Luciferase activity (%)

- Control
- LPS (1 ng/mL)
- LPS (1 ng/mL) + Isotype
- LPS (10 ng/mL)
- LPS (10 ng/mL) + TLR4 Ab

b) Luciferase activity (%)

- Control
- Pam3CSK4
- Pam3CSK4 + Isotype
- Pam3CSK4 + TLR4 Ab
- FSL-1
- FSL-1 + Isotype
- FSL-1 + TLR2 Ab

Luciferase activity (%)

- Control
- LPS (1 ng/mL)
- LPS (1 ng/mL) + Isotype
- LPS (10 ng/mL)
- LPS (10 ng/mL) + TLR4 Ab

IL-6 (ng/mL)

- Control
- LPS (1 ng/mL)
- LPS (1 ng/mL) + Isotype
- LPS (1 ng/mL) + TLR4 Ab

IL-6 (ng/mL)

- Control
- Pam3CSK4
- Pam3CSK4 + Isotype
- Pam3CSK4 + TLR4 Ab
- FSL-1
- FSL-1 + Isotype
- FSL-1 + TLR2 Ab

* p < 0.05
** p < 0.01

Graphs showing the effects of various treatments on luciferase activity and IL-6 levels.
Supplementary Figure 3

(a) Luciferase activity (%)
Control
Pam3CSK4
Pam3CSK4 + Isotype
Pam3CSK4 + TLR2 Ab
FSL-1
FSL-1 + Isotype
FSL-1 + TLR2 Ab

(b) Luciferase activity (%)
Control
Pam3CSK4
Pam3CSK4 + Isotype
Pam3CSK4 + TLR2 Ab
FSL-1
FSL-1 + Isotype
FSL-1 + TLR2 Ab

(c) IL-6 (ng/mL)
Control
LPS 1 ng/mL
LPS 1 ng/mL + Isotype
LPS 1 ng/mL + TLR4 Ab
LPS 10 ng/mL
LPS 10 ng/mL + Isotype
LPS 10 ng/mL + TLR4 Ab

(d) IL-6 (ng/mL)
Control
LPS 1 ng/mL
LPS 1 ng/mL + Isotype
LPS 1 ng/mL + TLR4 Ab
LPS 10 ng/mL
LPS 10 ng/mL + Isotype
LPS 10 ng/mL + TLR4 Ab
Supplementary Figure 4

Donor V683  Donor V695

a) MMP-3
   (55kDa)

b) MMP-9
   (92kDa)

MMP-2
   (72kDa)

Isotype Ab  Anti-TLR-4 Ab  Anti-TLR-2 Ab  Isotype Ab  Anti-TLR-4 Ab  Anti-TLR-2 Ab  Positive Control
Supplementary Figure 5

a) Anti-TRAM Ab
b) Anti-α-tubulin Ab

c) Ad C  AdTRAM\textsuperscript{DN}
  
  Anti-TRAM Ab
  Anti-α-tubulin Ab

d) Viability (MTT)

\begin{center}
\begin{tabular}{c|c|c}
\hline
Time (h) & Viability & % Uninfected \\
\hline
48 & 100 & \\
72 & 100 & \\
96 & 100 & \\
\hline
\end{tabular}
\end{center}

e) Luciferase activity

\begin{center}
\begin{tabular}{c|c}
\hline
Treatment & Luciferase activity \\
\hline
Control Ad & 100 \\
AdTRAM\textsuperscript{DN} & 25 * \\
\hline
\end{tabular}
\end{center}
Supplemental Figure 1

Phenotyping and cell survival analyses

(a) **Macrophages are the most abundant cells in the atheroma cell culture.** Atheroma cells were isolated and cultured for 72 hours. CD3⁺, CD68⁺ and ASMA⁺ cells were identified by single staining and FACS analysis. Data are shown as mean percentages of live events ± s.e.m. (n=16). Macrophages are the most represented cell type as compared to lymphocytes and SMC (***P = .0001; Wilcoxon Rank Sum Test).

(b) **Apoptosis in atheroma cell cultures** Apoptosis was assayed using annexin V after 72 hours in culture. Lymphocyte, macrophage and SMC-rich areas were identified by size and forward scatter. Data are shown as mean percentages ± s.e.m. (n=8). Lymphocytes underwent apoptosis to a greater extent in culture as compared to macrophages and smooth muscle cells (***P = .0001; Wilcoxon Rank Sum Test).
Supplemental Figure 2

(a-b) Neutralizing antibodies block TLR-4 and TLR-2 ligand-induced NFκB activity in human monocyte-derived macrophages. Monocyte-derived macrophages were infected with an adenovirus containing an NFκB luciferase reporter gene (AdNFκBluc). Subsequently cells were incubated with neutralizing anti-TLR4 (a) or anti-TLR2 (b) antibodies or Isotype control (final concentration of 25 μg ml⁻¹) for 30 minutes prior to addition of LPS (1 and 10 ng/mL; a), Pam3CSK4 (100 ng/mL; b) or FSL-1 (100 ng/mL; b). Mean and standard deviation of luciferase activity of triplicate cultures is shown and is representative of 3 independent experiments from unrelated donors. Treatment with TLR-4 (a) or TLR-2 (b) blocking antibody inhibits ligand-induced NFκB activation (**P=.001 compared to Isotype-treated cells; Wilcoxon Rank Sum test).

(c-d) Neutralizing antibodies block TLR-4 and TLR-2 ligand-induced IL-6 production in human monocyte-derived macrophages. Monocyte-derived macrophages were incubated with neutralizing anti-TLR-4 (c) or anti-TLR-2 (d) antibodies and Isotype control (final concentration of 25 μg ml⁻¹) for 30 minutes prior to treatment with LPS (1 and 10 ng/mL; c), Pam3CSK4 (100 ng/mL; d) or FSL-1 (100 ng/mL; c). Supernatants were assayed by ELISA for IL-6. Mean and standard deviation of cytokine production of triplicate cultures is shown and is representative of 3 independent experiments from unrelated donors. Treatment with TLR-4 (c) or TLR-2 (d) blocking antibody inhibits ligand-induced IL-6 production (*P<.05 compared to Isotype-treated cells; Wilcoxon Rank Sum test).
Supplemental Figure 3

(a-b) Neutralizing antibodies block TLR-4 and TLR-2 ligand-induced NFκB activity in HUVECs. HUVECs were infected with an adenovirus containing an NFκB luciferase reporter gene (AdNFκBluc). Subsequently cells were incubated with neutralizing anti-TLR4 (a) or anti-TLR2 (b) antibodies or Isotype control (final concentration of 25 μg ml⁻¹) for 30 minutes prior to addition of LPS (1 and 10 ng/mL; a), Pam3CSK4 (100 ng/mL; b) or FSL-1 (100 ng/mL; b). Mean and standard deviation of luciferase activity of triplicate cultures is shown and is representative of 3 independent experiments from unrelated donors. Treatment with TLR-4 (a) or TLR-2 (b) blocking antibody inhibits ligand-induced NFκB activation (***P=.001 compared to Isotype-treated cells; Wilcoxon Rank Sum test).

(c-d) Neutralizing antibodies block TLR-4 and TLR-2 ligand-induced IL-6 production in HUVECs. HUVECs were incubated with neutralizing anti-TLR-4 (c) or anti-TLR-2 (d) antibodies and Isotype control (final concentration of 25 μg ml⁻¹) for 30 minutes prior to treatment with LPS (1 and 10 ng/mL; c), Pam3CSK4 (100 ng/mL; d) or FSL-1 (100 ng/mL; d). Supernatants were assayed by ELISA for IL-6. Mean and standard deviation of cytokine production of triplicate cultures is shown and is representative of 3 independent experiments from unrelated donors. Treatment with TLR-4 (c) or TLR-2 (d) blocking antibody inhibits ligand-induced IL-6 production (*P<.05 compared to Isotype-treated cells; Wilcoxon Rank Sum test).
Supplemental Figure 4

Zymography demonstrates reduction of MMP levels in atheroma cell cultured in the presence of TLR-2 neutralizing antibody Atheroma cell supernatants were collected at 96 hours after culture in the presence of neutralizing antibodies for TLR-4 or TLR-2 or Isotype control. Supernatants were then run on either 0.1% casein 12% acrylamide gel (a) or 0.1% gelatin 10% acrylamide gel (b). Incubation of atheroma cells with TLR-2 antibody reduced MMP-2, -3 and -9 content in the supernatants. Two representative donors out of 6 are shown.
Supplemental Figure 5

(a-c) Successful adenoviral gene transfer targeting TRAM in cells isolated from human atherosclerotic tissue. Successful adenoviral infection was monitored in each experiment via a GFP tag contained in the adenoviruses. One representative experiment is shown (total n=6); Bright light field (a) Direct immunofluorescence (b); magnification 20x. (c) Expression of the transgenes of interest was monitored by Immunoblotting for TRAM and α–tubulin. Representative blots are shown (total n=3). Successful overexpression of the transgenes compared to endogenous levels was demonstrated in all occasions.

d) Infection with AdTRAM^{DN} did not affect cell viability in culture. Cellular viability was assessed by MTT. Data are shown as mean ± s.e.m. (n = 10). Values are % relative to untreated cells. There was no significant difference in viability between uninfected and adenovirus-infected cells after infection (P>.05; Rank ANCOVA).

e) TRAM is involved in NFκB activation in human atheroma cell cultures. NFκB activation after 48 hours in culture with indicated adenoviruses/blockers was monitored via a NFκB luciferase reporter assay. Data are shown as mean ± s.e.m. (n = 3). Values are % of luciferase activity relative to uninfected cells. No significant difference was observed between uninfected cells and control adenovirus-infected cells (P>.05). Significant inhibition of NFκB was achieved with AdTRAM^{DN} versus Control Ad-infected cells (*P<.05; Wilcoxon Rank Sum Test).