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Research Article **Cardiology**

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Epidemiologic evidence has established a relationship between microbial infection and atherosclerosis. Mammalian TLRs provide clues on the mechanism of this inflammatory cascade. TLR2 has a large ligand repertoire that includes bacterial-derived exogenous and possibly host-derived endogenous ligands. In atherosclerosis-susceptible *low-density lipoprotein receptor–deficient* ($Ldlr^{-/-}$) mice, complete deficiency of TLR2 led to a reduction in atherosclerosis. However, with BM transplantation, loss of TLR2 expression from BM-derived cells had no effect on disease progression. This suggested that an unknown endogenous TLR2 agonist influenced lesion progression by activating TLR2 in cells that were not of BM cell origin. Moreover, with intraperitoneal administration of a synthetic TLR2/TLR1 agonist, Pam3CSK4, disease burden was dramatically increased in $Ldlr^{-/-}$ mice. A complete deficiency of TLR2 in $Ldlr^{-/-}$ mice, as well as a deficiency of TLR2 only in BM-derived cells in $Ldlr^{-/-}$ mice, led to striking protection against Pam3CSK4-mediated atherosclerosis, suggesting a role for BM-derived cell expression of TLR2 in transducing the effects of an exogenous TLR2 agonist. These studies support the concept that chronic or recurrent microbial infections may contribute to atherosclerotic disease. Additionally, these data suggest the presence of host-derived endogenous TLR2 agonists.

Introduction

Complications from atherosclerosis represent a major cause of morbidity and mortality in Western society (1). The inflammatory nature of this disease process is widely accepted; however, the precise components of the atherogenic proinflammatory cascade remain controversial (2). Epidemiologic evidence has demonstrated a relationship between microbial infection and disease, with experimental studies suggesting a causal role (3–6). The recent discovery of mammalian TLRs as principal sensors of the innate immune system provides a mechanistic link between infection, inflammation, and atherosclerosis (7). Additionally, the possibility of endogenous ligand activation of TLRs provides sterile inflammation links to atherosclerosis susceptibility (8, 9).

Initial evidence suggesting a link between atherosclerosis and TLR activation can be found in epidemiologic studies linking bacterial infection with atherosclerotic disease (4, 5, 10). Such studies have demonstrated a link between bacterial infection and disease. Additionally, studies have demonstrated common infectious agents, such as *Chlamydia pneumoniae, Porphyromonas gingivalis* and *Helicobacter pylori*, to either be blood-borne or localized within atherosclerotic lesions (11–14). These particular microorganisms are common etiologic agents of chronic infection in the human population and can provide for an ample supply of TLR ligands. A link between atherosclerosis and TLRs is found in studies that demonstrated atherosclerotic lesion cell expression of TLR1, 2, and 4 in humans (15, 16). Experimental studies identified a role for TLR4 in atherosclerosis (17, 18) as well as the common TLR downstream signaling adaptor molecule myeloid differentiation factor 88 (MyD88) (18, 19). For example, Michelsen et al. found

that a loss of TLR4 or MyD88 reduces disease severity in atherosclerosis-prone *apolipoprotein E–deficient* ($Apoe^{-/-}$) mice (18). Additionally, a recent study has demonstrated a proatherogenic effect of local TLR2 activation (20). However, the role of TLR2 in atherosclerosis is controversial (21).

TLR2 detects a large range of microbial components, such as gram-positive–derived lipoteichoic acid, bacterial lipoproteins, and zymosan (22). Of the 11 characterized TLRs, TLR2 is unique by virtue of its ability to heterodimerize with TLR1 or TLR6 (23), resulting in a relatively broad ligand specificity (24, 25). The recent discovery of CD36 as a coreceptor for TLR2 raises the prospect that a novel proinflammatory pathway exists between endogenously derived lipids and activation of innate immunity (26). Two outstanding questions remain: Does TLR2 play an important role in atherosclerosis, either in the presence or absence of exogenous ligands, and what innate immune "effector" cells transduce the proinflammatory events initiated by TLR2 activation?

Previously reported in vitro studies suggest that enhanced endothelial TLR2 expression and activation may occur at areas of disturbed blood flow, such as the areas of lesion predilection within the aortic tree and heart (27). This suggests that TLR2 expression may promote atherosclerosis in cells that are not of BM origin, such as endothelial cells, and thus may contribute to the proinflammatory phenotype of activated endothelial cells.

We sought to determine the role of in vivo activation of TLR2 in atherosclerosis, utilizing the atherosclerosis-prone *low-density lipoprotein receptor–deficient* ($Ldlr^{-/-}$) mouse. Our aim was to clarify the impact of cellular TLR2 expression and activation in atherosclerosis and to determine if cells of BM origin were necessary to transduce the proinflammatory signals that result from TLR2 activation.

In this report, we provide evidence of a solid relationship between TLR2 and atherosclerosis. Our data demonstrate that, in the absence of any known exogenous TLR2 agonist, complete deficiency of TLR2 in $Ldlr^{-/-}$ mice led to a reduction in atherosclerosis, whereas loss of TLR2 expression in BM-derived cells did not have an impact on disease. This suggested that an unknown endogenous
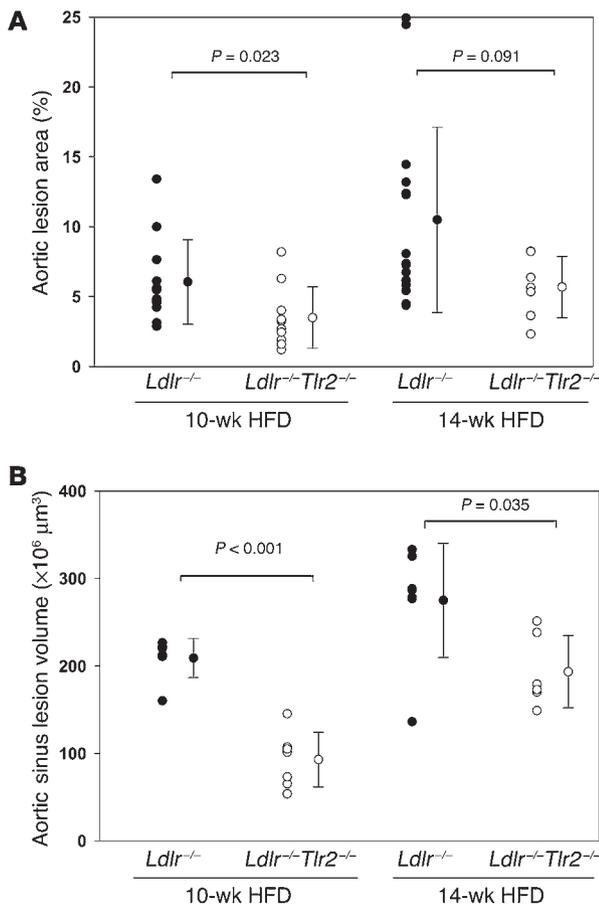

Figure 1
Measurement of atherosclerosis in male *Ldlr⁻/⁻* and *Ldlr⁻/⁻Tlr2⁻/⁻* mice after 10 or 14 weeks of consuming an HFD. (**A**) Aortic lesion area was calculated as fraction of total aorta covered by lesion. (**B**) Aortic sinus lesion volume was calculated from an integration of the cross-sectional areas of the lesion in the proximal 500 μm of the sinus. Aortic lesion area and aortic valve lesion volume were significantly decreased in *Ldlr⁻/⁻Tlr2⁻/⁻* mice.

Analysis of aortic lesion area and aortic valve lesion volume revealed a significant decrease in disease in *Ldlr⁻/⁻Tlr2⁻/⁻* mice, with an almost 50% decrease in aortic atherosclerosis (at both time points) and a 55% and 30% decrease in aortic valve lesion volume after 10 and 14 weeks, respectively (Figure 1, A and B).

BM-derived cell expression of TLR2. Due to the pivotal role that macrophages serve in atherogenesis, we hypothesized that the absence or presence of macrophage TLR2 expression would also have an impact on atherosclerosis. Utilizing BM transplantation (BMT), *Ldlr⁻/⁻* mice were generated that lacked TLR2 expression in their BM-derived cells. Similarly, *Ldlr⁻/⁻Tlr2⁻/⁻* mice were generated that expressed TLR2 only in their BM cells.

In the first BMT, 20-week-old female *Ldlr⁻/⁻* mice were reconstituted with BM from donor mice lacking TLR2 (*Tlr2⁻/⁻*) (*n* = 17) or their respective wild-type controls (*Tlr2⁺/⁺*) (*n* = 21). After 4 weeks of recovery, animals were fed the HFD for 16 weeks, after which they were sacrificed and their aortae and hearts harvested. *Ldlr⁻/⁻* mice receiving *Tlr2⁻/⁻* BM had similar body weight gain as those receiving *Tlr2⁺/⁺* marrow throughout the study (Supplemental Figure 2A). Both groups also had a similar increase in plasma cholesterol upon an HFD, with plasma cholesterol increasing at each time point thereafter. Nevertheless, there were differences in plasma cholesterol between these groups at the 4- and 16-week time points (Supplemental Figure 2B). In *Ldlr⁻/⁻Tlr2⁻/⁻* mice receiving *Tlr2⁺/⁺* (*n* = 14) or *Tlr2⁻/⁻* (*n* = 16) BM, we also observed no changes in weight gain between the 2 groups with consistent growth observed throughout the 16-week feeding period (Supplemental Figure 2C). Plasma cholesterol levels in these 2 groups were similar throughout the study (Supplemental Figure 2D).

Surprisingly, quantitation of atherosclerosis revealed no impact of BM-derived cellular expression of TLR2 on aortic sinus lesion
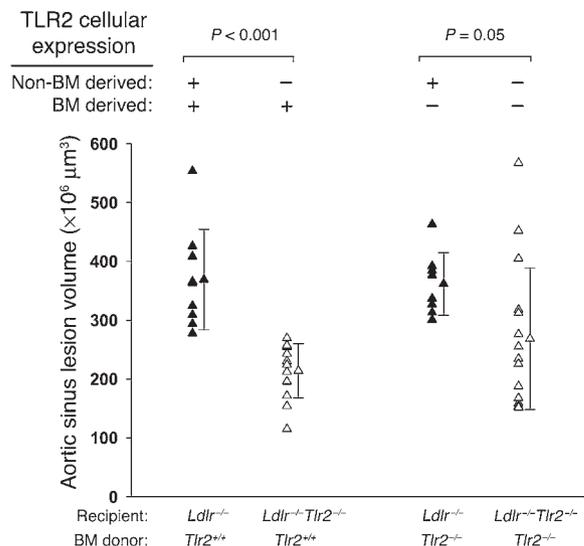
TLR2 agonist, or agonists, had an impact on atherosclerotic disease. Moreover, with i.p. administration of a synthetic TLR2/TLR1 agonist, N-palmitoyl-(S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl] (Pam3)/Cys-Ser-Lys 4 (CSK4) (Pam3CSK4; referred to herein as Pam3), disease burden was dramatically increased in *Ldlr⁻/⁻* mice. A complete deficiency of TLR2 in *Ldlr⁻/⁻* mice as well as a deficiency of TLR2 only in BM-derived cells in *Ldlr⁻/⁻* mice led to a dramatic protection against this Pam3-mediated atherosclerosis.
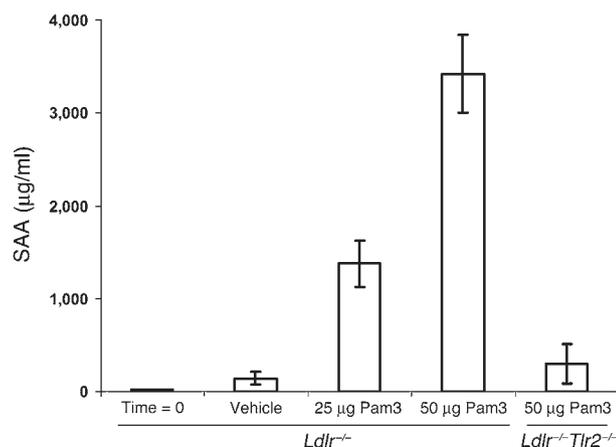
Results

Total body deficiency of TLR2. To determine the role of TLR2 in atherosclerosis, *Tlr2⁻/⁻* mice were crossed with atherosclerosis-prone *Ldlr⁻/⁻* mice to generate double-knockout *Ldlr⁻/⁻Tlr2⁻/⁻* mice. Twelve-week-old male mice were fed a high-fat diet (HFD) for 10 and 14 weeks. Relative to *Ldlr⁻/⁻* mice (*n* = 27), *Ldlr⁻/⁻Tlr2⁻/⁻* mice (*n* = 17) had reduced total plasma cholesterol levels and increased body weight throughout 10 or 14 weeks of an HFD (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI25482DS1). Averaged over the HFD feeding period, the reduction in total plasma cholesterol in *Ldlr⁻/⁻Tlr2⁻/⁻* mice was 16% less relative to *Ldlr⁻/⁻* mice (1242 ± 50 vs. 1485 ± 34 mg/dl, *P* < 0.05).

Figure 2
Measurement of heart aortic sinus atherosclerosis volume after 16 weeks of HFD feeding in female *Ldlr⁻/⁻* and *Ldlr⁻/⁻Tlr2⁻/⁻* mice that underwent BM reconstitution with BM from *Tlr2⁺/⁺* or *Tlr2⁻/⁻* donors. Regardless of BM cell genotype, a loss of TLR2 on non-BM–derived cells significantly reduced disease.

in response to i.p. administration of Pam3. Injection of Pam3 in the *Ldlr*^{−/−}*Tlr2*^{−/−} mice was similar to the vehicle injections in *Ldlr*^{−/−} mice, confirming the specificity of the Pam3 preparation for TLR2. Throughout the study, all mice experienced similar rates of weight gain (Supplemental Figure 3A). After 4 weeks of consuming the HFD and weekly injections, total plasma cholesterol was significantly increased in all groups, from 240 to 1,330 (vehicle), 270 to 1,750 (25 μg of Pam3) and 260 to 1,340 mg/dl (50 μg of Pam3) (Supplemental Figure 3B). By the eighth and twelfth weeks, the Pam3 groups had similar plasma cholesterol levels, which were

volume in *Ldlr*^{−/−} recipients (Figure 2; *Ldlr*^{−/−} recipient, *Tlr2*^{+/+} donor vs. *Ldlr*^{−/−} recipient, *Tlr2*^{−/−} donor) or in *Ldlr*^{−/−}*Tlr2*^{−/−} BM recipients (Figure 2; *Ldlr*^{−/−}*Tlr2*^{−/−} recipient, *Tlr2*^{+/+} donor vs. *Ldlr*^{−/−}*Tlr2*^{−/−} recipient, *Tlr2*^{−/−} donor). This BMT study was also performed in male mice, and similar results were obtained. Importantly, comparison of groups with only changes in non-BM expression of TLR2 duplicated our finding in the male double-mutant mice that non-BM cell expression of TLR2 modulated atherosclerosis. For example, significant decreases were observed in atherosclerosis in mice that lacked TLR2 expression in non-BM cells but expressed TLR2 in BM-derived cells (Figure 2; *Ldlr*^{−/−} recipient, *Tlr2*^{+/+} donor vs. *Ldlr*^{−/−}*Tlr2*^{−/−} recipient, *Tlr2*^{+/+} donor) or lacked TLR2 in BM-derived cells (Figure 2; *Ldlr*^{−/−} recipient, *Tlr2*^{−/−} donor vs. *Ldlr*^{−/−}*Tlr2*^{−/−} recipient, *Tlr2*^{−/−} donor). The data for total aortic lesion area showed the same trends as the data for aortic sinus lesion volume discussed above.

Exogenous agonist activation of TLR2. Studies were performed to evaluate the impact of an exogenous TLR2 agonist on atherosclerosis. Twelve-week-old mice received weekly i.p. injections of Pam3, a synthetic TLR2 agonist that mimics the triacylated amino terminus of bacterial lipoproteins and activates TLR2/TLR1 heterodimers (24). Twenty-two female *Ldlr*^{−/−} mice were split into 3 groups that received weekly i.p. injections of vehicle, 25 μg of Pam3, or 50 μg of Pam3. An ELISA measurement of the acute phase protein, serum amyloid A (SAA), was performed 24 hours after initiation of the HFD and i.p. injections (Figure 3). The vehicle injection group experienced a 9-fold increase in SAA (16 ± 3 vs. 140 ± 68, $P < 0.05$). Relative to the increase seen with vehicle injections, we measured a 10-fold and 24-fold increase with 25 and 50 μg of Pam3, respectively (1,380 ± 250 vs. 3,420 ± 420 μg/ml SAA, $P < 0.05$). This demonstrated a systemic inflammatory reaction
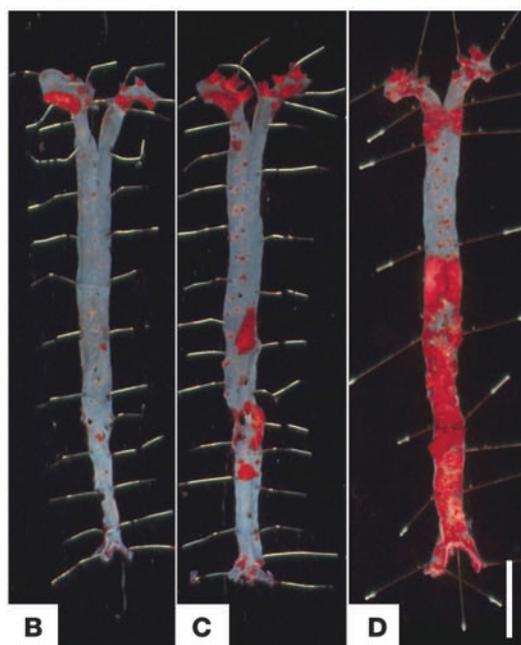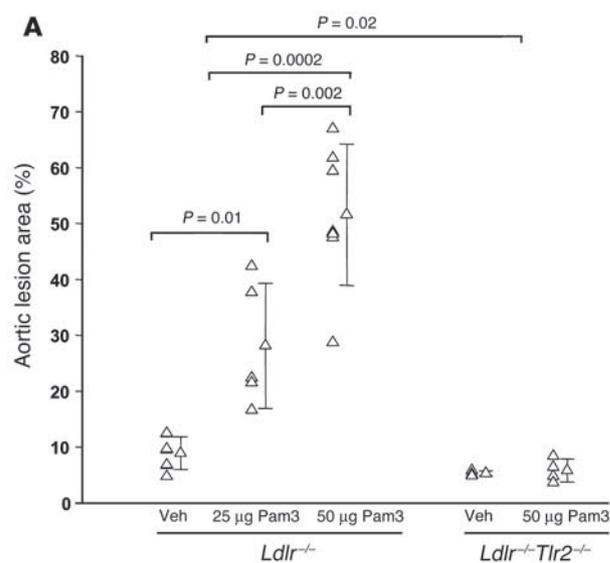
Figure 4
Measurement of aortic atherosclerosis in Pam3-exposed mice. (**A**) Aortic atherosclerosis expressed as a fraction of total area after 12 weeks of an HFD and weekly i.p. injections of vehicle (veh) or Pam3 in female *Ldlr*^{−/−} and *Ldlr*^{−/−}*Tlr2*^{−/−} mice. (**B**–**D**) Representative aortae from *Ldlr*^{−/−} mice in the following groups: vehicle (**B**), 25 μg Pam3 (**C**), and 50 μg Pam3 (**D**). A dose-response effect of Pam3 administration and lesion development was observed. Profuse abdominal aortic atherosclerosis was observed in mice exposed to 50 μg Pam3. Lesion severity in TLR2-deficient animals was not affected by Pam3. Scale bar: 0.5 cm.
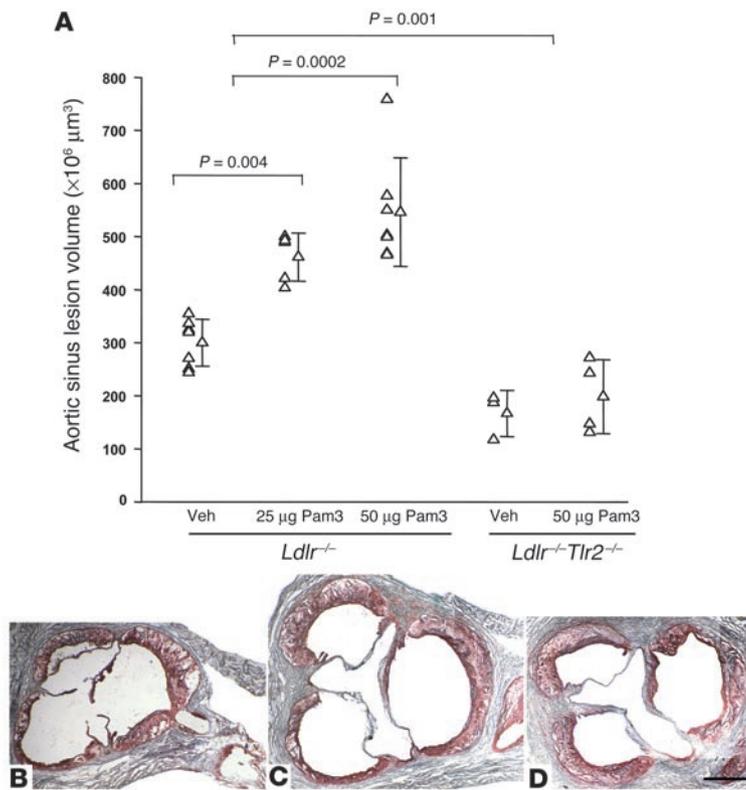
Figure 5
Measurement of heart aortic sinus atherosclerosis volume in Pam3-exposed mice. (**A**) Heart aortic sinus atherosclerosis volume after 12 weeks of an HFD and weekly i.p. injections of Pam3 in female $Ldlr^{-/-}$ and $Ldlr^{-/-}Tlr2^{-/-}$ mice. (**B–D**) Representative cross-sections of the aortic sinus from $Ldlr^{-/-}$ mice of the following groups: vehicle (**B**), 25 μg Pam3 (**C**), and 50 μg Pam3 (**D**). A dose-response effect of Pam3 administration and lesion development was observed. Lesion severity in TLR2-deficient animals was not affected by Pam3. Scale bar: 0.5 mm.

deficient in TLR2 ($Ldlr^{-/-}Tlr2^{-/-}$) exhibited no changes in aortic or aortic sinus atherosclerosis and confirmed the specificity of Pam3 for TLR2-mediated cell activation (Figures 4 and 5). Additionally, comparison of aortic and aortic sinus atherosclerosis in $Ldlr^{-/-}$ and $Ldlr^{-/-}Tlr2^{-/-}$ animals receiving vehicle injections revealed the atheroprotective effect of complete TLR2 deficiency, as demonstrated previously. Finally, a comparison of the trends observed in male cohorts (Figure 1) and female cohorts (Figures 4 and 5; vehicle controls) confirmed that the atheroprotection seen with TLR2 deficiency was not sex-dependent.

Utilizing BMT, the contribution of macrophage TLR2 expression was ascertained in the setting of exogenous ligand activation of TLR2 by Pam3. In this BMT study, 40 female $Ldlr^{-/-}$ mice were split equally into 2 groups, with 1 group receiving BM from wild-type $Tlr2^{+/+}$ mice and the other from $Tlr2^{-/-}$ mice, similar to our previous BMT studies. Mice in each group were further subdivided into groups receiving weekly i.p. injections of 50 μg Pam3 or vehicle.

Among the 4 groups (wild-type vs. $Tlr2^{-/-}$ BM recipients ± 50 μg Pam3), the wild-type $Ldlr^{-/-}$ recipients receiving Pam3 experienced a slight decrease in body weight toward the end of the treatment period (Supplemental Figure 4A) whereas the $Tlr2^{-/-}$ recipients receiving Pam3 demonstrated slight increases in body weight (Supplemental Figure 4C). Animals with BM-derived cell expression of TLR2 demonstrated large increases in atherosclerosis with Pam3 administration (Table 1 and Figures 6, A–E, and 7, A–E). In these groups, aortic atherosclerosis increased by 280% and aortic sinus lesion volume increased by 90% relative to the vehicle con-

reduced relative to the vehicle group: 1,110 (vehicle), 900 (25 μg of Pam3), and 850 mg/dl (50 μg Pam3) ($P < 0.05$). Averaging each group's plasma cholesterol during the HFD feeding revealed a slight cholesterol-lowering effect from Pam3 exposure. Additionally, 7 female $Ldlr^{-/-}Tlr2^{-/-}$ mice were used as negative controls to test for the specificity of Pam3 for TLR2. $Ldlr^{-/-}Tlr2^{-/-}$ mice receiving 50 μg of Pam3 exhibited no differences in body weight or total plasma cholesterol levels relative to vehicle-injected $Ldlr^{-/-}Tlr2^{-/-}$ mice.

Analysis of aortic atherosclerosis demonstrated a significant increase in lesion areas with Pam3 administration. Relative to the vehicle-injected group, a dose-response effect of Pam3 was observed in aortic atherosclerosis, with significant increases in lesion severity (relative to the vehicle group) of 220% and 480% observed in the 25 and 50 μg Pam3 groups, respectively (Figure 4A). Pam3 administration resulted in a peculiar distribution of lesion development with profuse abdominal atherosclerosis observed in the 50 μg Pam3 group and sporadic abdominal lesions in the 25 μg Pam3 group (Figure 4, C and D). A similar dose-response effect was observed with aortic sinus lesion volume, with an increase in lesion volume (relative to the vehicle group) of 54% and 82% in the 25 μg Pam3 and 50 μg Pam3 groups, respectively ($P < 0.05$) (Figure 5A). Mice

Table 1
Summary of TLR2 agonist studies

| | TLR2 cellular expression | | | | | |
Aortic lesion	BM derived	Non-BM derived	Vehicle	50 μg Pam3	% change	*P* value
$Ldlr^{-/-}$, no BMT	+	+	8.9%	51.6%	479%	0.0006
$Ldlr^{-/-}$, WT BM	+	+	7.3%	27.8%	279%	0.0209
$Ldlr^{-/-}$, $Tlr2^{-/-}$ BM	–	+	5.5%	7.7%	40%	0.0211
$Ldlr^{-/-}Tlr2^{-/-}$, no BMT	–	–	5.3%	5.8%	10%	0.6829
Lesion volume						
$Ldlr^{-/-}$, no BMT	+	+	300	546	82%	0.0006
$Ldlr^{-/-}$, WT BM	+	+	220	416	89%	0.0003
$Ldlr^{-/-}$, $Tlr2^{-/-}$ BM	–	+	223	269	21%	0.3913
$Ldlr^{-/-}Tlr2^{-/-}$, no BMT	–	–	167	198	19%	0.6286

Aortic lesion values are given as a percentage of the total aortic area that contained lesions. Lesion volume data are reported as volume of aortic sinus lesion in proximal 500 μm of sinus × 10^6 μm^3.
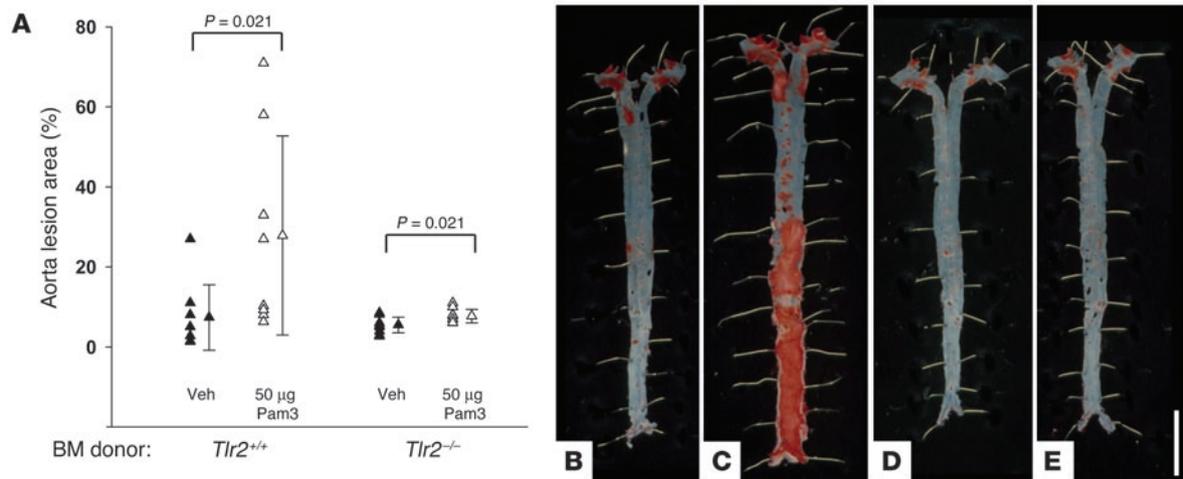
Figure 6
Measurement of aortic atherosclerosis in Pam3-exposed TLR2 chimeric mice. (**A**) Aortic atherosclerosis expressed as a percentage of total area after 12 weeks of an HFD and weekly i.p. injections of Pam3 in female *Ldlr*−/− mice that underwent BM reconstitution from *Tlr*+/+ or *Tlr2*−/− donor mice. (**B**–**E**) Representative aortae from *Ldlr*−/− recipient mice of the following groups: *Tlr2*+/+ BM donor, vehicle injection (**B**); *Tlr2*+/+ BM donor, 50 μg Pam3 per week (**C**); *Tlr2*−/− BM donor, vehicle injection (**D**); and *Tlr2*−/− BM donor, 50 μg Pam3 per week (**E**). Pam3 administration increased lesion severity in animals expressing TLR2 in their BM-derived cells resulting in profuse abdominal aortic atherosclerosis. Slight increases in lesion severity were observed in *Tlr2*−/− recipient animals receiving Pam3. Scale bar: 0.5 cm.

trols (Table 1). These profound increases in disease were similar to those observed in the non-BMT agonist injection studies (Table 1), with profuse abdominal atherosclerosis observed with Pam3 administration (Figure 6C). A comparison of the vehicle control groups in this study duplicated the observation of our previous BMT study that BM-derived cell expression of TLR2 did not modulate atherosclerosis in the absence of an exogenous agonist (Figures 6, A, B, and D, and 7, A, B, and D). In the absence of BM-derived cell expression of TLR2, Pam3 administration had only a slight effect on increasing atherosclerosis in the aorta (Table 1 and

Figure 6, A, D, and E) and no effect on aortic sinus lesion volume (Table 1 and Figure 7, A, D, and E).

Lipoprotein cholesterol distribution. Because TLR2 deficiency or activation may change the lipoprotein distribution of plasma cholesterol, the distribution of total cholesterol within the major lipoprotein fractions was analyzed by fast protein liquid chromatography (FPLC). FPLC was performed on plasma samples obtained from mice 8 weeks after initiation of the HFD. Equal volumes of samples were pooled from 4–8 representative mice per group. As previously reported (28), the vast majority of total plasma cholesterol was
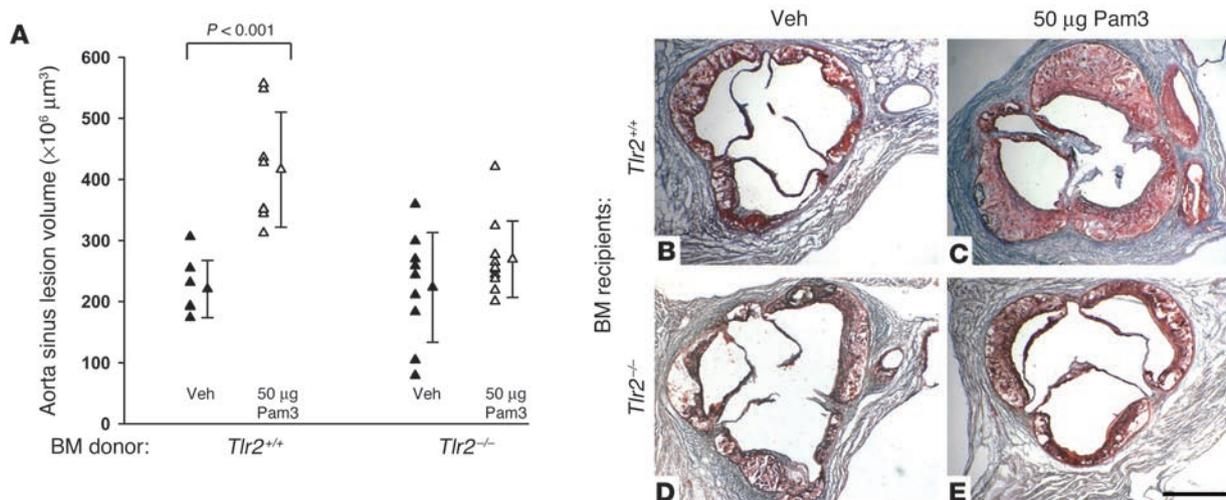

Figure 7
Measurement of heart aortic sinus atherosclerosis volume in Pam3-exposed TLR2 chimeric mice. (**A**) Heart aortic sinus atherosclerosis after 12 weeks of an HFD and weekly i.p. injections of Pam3 in female *Ldlr*−/− mice that underwent BM reconstitution from *Tlr*+/+ or *Tlr2*−/− donor mice. (**B**–**E**) Representative aortic sinus cross-sections from *Ldlr*−/− mice of the following groups: *Tlr2*+/+ BM donor, vehicle injections (**B**); *Tlr2*+/+ BM donor, 50 μg Pam3 injections (**C**); *Tlr2*−/− BM donor, vehicle injections (**D**); and *Tlr2*−/− BM donor, 50 μg Pam3 injections (**E**). Pam3 administration increased lesion severity only in animals expressing TLR2 in their BM-derived cells. Scale bar: 0.5 mm.
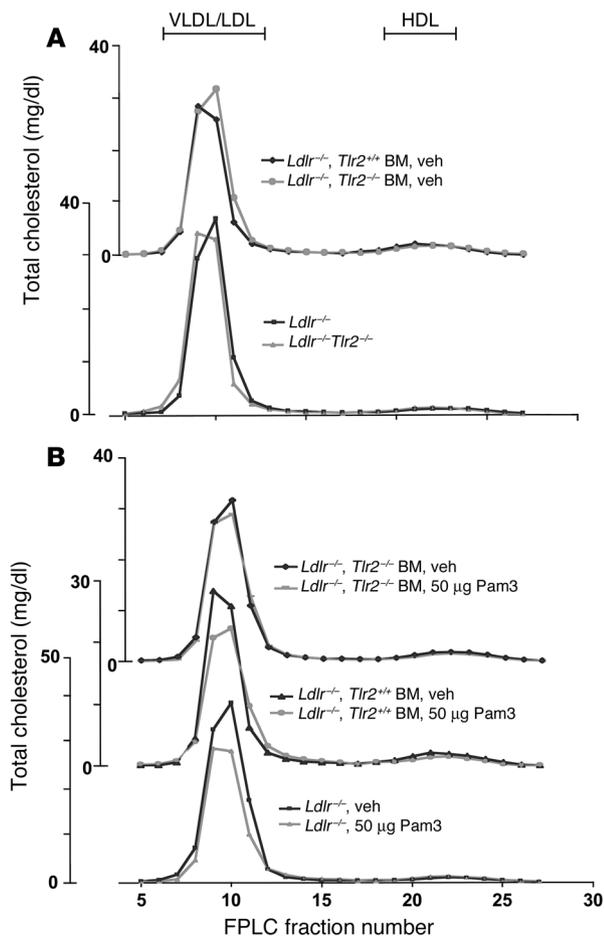
Figure 8
Plasma lipoprotein cholesterol distribution. Pooled plasma samples taken from representative mice after 8 weeks of consumption of the HFD were fractionated by FPLC, and total cholesterol was measured. (**A**) Complete or BM cell–derived loss of TLR2 did not alter the distribution of cholesterol relative to control. (**B**) Administration of 50 μg Pam3 also did not have an impact on the distribution of cholesterol relative to control mice given the vehicle injections.

with some studies (including our own) demonstrating expression (29, 30) and some not (31, 32). Human studies have established that TLR2 expression was elevated in human atherosclerotic lesions (16), and peptidoglycan (a component of gram-positive bacteria) was found to be associated with arterial cells located in atherosclerotic plaques (33).

Our studies suggest that endogenous agonist(s) for TLR2 have an impact on atherosclerotic disease progression. With the complete absence of TLR2, both in the non-BMT and BMT studies, we observed a consistent reduction in lesion area in the absence of any known exogenous agonist. This is solid evidence that TLR2 activation contributed to lesion development in the hyperlipidemic *Ldlr−/−* mice. This is consistent with the observation that MyD88 deficiency leads to a reduction of atherosclerosis in *ApoE−/−* mice (18, 19) since downstream signaling of TLR2 is dependent upon MyD88 (34). A recent report that identified the scavenger receptor CD36 as a coreceptor and activator of TLR2 suggests a mechanistic link between lipids and TLR2-mediated inflammation (26). The endogenous ligands for TLR2 and its heterodimers TLR1 and 6 and/or CD36 could include any of the diverse lipids generated from oxidative modification of lipoproteins and necrotic or apoptotic cells. Nevertheless, the possibility that an unknown endemic exogenous TLR2 agonist also participated in the disease outcome in these studies cannot be ruled out.

The proatherogenic effects of administration of a known exogenous TLR2 agonist were clear. Profound increases in lesion development were observed with i.p. administration of Pam3. Interestingly, there were no significant differences in body weight in the *Ldlr−/−* mice receiving 25 or 50 μg of Pam3, suggesting preservation of overall health in this model despite their advanced atherosclerosis. The peculiar distribution of lesion development with Pam3 administration in the non-BMT and BMT groups (Figures 4, C and D, and 6C) suggests that peritoneal-derived inflammatory mediators influence lesion development in these animals. However, these mice exhibited accelerated lesion development in regions of the vasculature not directly in contact with the peritoneal cavity (such as the aortic arch and sinus). Because this model involved i.p. injections, it was possible that the effects of Pam3 on atherogenesis were also mediated by generalized systemic proinflammatory responses to Pam3 exposure, such as secondary inflammatory responses elicited after the peritoneal injection of Pam3. SAA measurements established the systemic inflammatory nature of the i.p. injections of Pam3 (Figure 3).

Our BMT studies demonstrated a critical role of BM-derived cell expression of TLR2 in transducing the proinflammatory effects of Pam3. Activation of TLR2 on BM-derived cells identified a potentially important role of macrophage TLR2 expression. Coupled with our demonstration of the importance of non-BM–derived expression of TLR2, a model of TLR2 expression and activation in atherosclerosis can be hypothesized. Perhaps early on in lesion progression, endothelial expression of TLR2 contributes to

located within the VLDL/LDL lipoprotein fractions. No changes were detected in the cholesterol distribution within the lipoprotein fractions from each group of mice (Figure 8, A and B).

Discussion

In this report, we establish a clear role for TLR2 in modulating the severity of experimental atherosclerosis. Complete loss of this receptor resulted in decreased lesion size whereas systemic exposure to a TLR2 ligand dramatically increased lesion severity. With BMT, we demonstrated the contribution of cellular TLR2 expression in lesion development. These data illustrate that, in the absence of a known exogenous agonist, loss of TLR2 expression on cells *not* of BM origin (such as endothelial cells) reduced atherosclerosis whereas BM–derived cell expression of TLR2 had no impact on disease. This was consistent with the idea that TLR2 expression on endothelial cells (or other non-BM–derived cells, such as vascular smooth muscle cells or fibroblasts) at sites of disease predilection contribute to the proinflammatory phenotype of atherosclerosis. Our previously reported data demonstrate that upregulation of human aortic endothelial TLR2 expression occurred in endothelial cells exposed to disturbed flow, and this provides further support for a relationship between endothelial TLR2 expression and atherosclerosis (27).

Previous studies have not established a clear role for TLR2 in atherosclerosis, with the majority of TLR studies in atherosclerosis focused on TLR4. Experimental data linking endothelial cell TLR2 expression and atherosclerosis remain controversial (21),

endothelial cell activation at lesion-prone sites in the vasculature. This could promote macrophage infiltration, with subsequent macrophage TLR2 activation (via any number of endogenous or exogenous agonists), exacerbating the proinflammatory milieu within the lesion.

These studies established that an injected exogenous TLR2/1 agonist exacerbated atherosclerosis and, therefore, provide insight into a potential mechanism by which recurrent microbial infections might influence disease severity (35, 36). Furthermore, we demonstrated a critical role of TLR2 expression by cells *not* of BM origin in enhancement of atherosclerotic disease severity in the absence of a known exogenous agonist. This is suggestive of endogenous TLR2 agonists, such as lipid-derived CD36 ligands. Given the spectrum of putative ligands for TLR2 and the incidence of chronic microbial infection, our data provide insight into a potential role for TLR2 activation in the exacerbation of atherosclerotic disease.

Methods

Animals. $Ldlr^{-/-}$ mice backcrossed onto a C57BL/6 background were purchased from The Jackson Laboratory and bred in house. $Tlr2^{-/-}$ mice were kindly provided by Tularik Inc. $Tlr2^{-/-}$ mice were backcrossed into a C57BL/6 background, and after 7 generations double-mutant mice ($Ldlr^{-/-}Tlr2^{-/-}$) were generated by crossing C57BL/6J-$Tlr2^{-/-}$ mice with $Ldlr^{-/-}$ mice ($Ldlr^{-/-}Tlr2^{-/-}$). $Tlr2^{-/-}$ genotyping was performed via PCR. In brief, DNA from tail clippings was amplified by 33 cycles of 45 seconds at 94°C, 45 seconds at 65°C, and 2 minutes extension at 72°C using specific primer pairs. The primers used for TLR2 DNA detection were designed to bind in the regions just upstream and within the neomycin insert (NEO) cassette inserted into the TLR2 gene. The TLR2 forward primer (5′-TTGGATAAGTCTGATAGCCTTGCCTCC-3′) and reverse primers (TLR2 5′-GTTTAGTGCCTGTATCCAGTCAGTGCG-3′ and NEO cassette 5′-ATCGCCTTCTATCGCCTTCTTGACGAG-3′) were synthesized by Operon. The PCR products were separated in 3% agarose gels and visualized with ethidium bromide. $Ldlr^{-/-}$ genotyping was performed as previously described (37).

The mice were weaned at 4 weeks and fed ad libitum a standard mouse chow diet (Harlan Teklad 7019). Mice used for the BMT studies were between 16 and 20 weeks old when they were initially fed the HFD, which contained 1.25% cholesterol, 15.8% fat, and no cholate (Harlan Teklad 94059). Mice in the non-BMT studies were fed the HFD diet at 12–15 weeks of age, after which they were fed the atherosclerotic diet for 10 or 14 weeks. Mice were fasted periodically and weighed and venous blood was drawn from the retro-orbital sinus. Plasma was isolated and total cholesterol levels were measured by a colorimetric enzymatic method (Thermo Electron Corp.). Lipoproteins were separated by FPLC on a Superdex 200 column (Pharmacia Corp.). A pool of plasma (0.1 ml) from representative mice was filtered and applied to 2 1 × 30 cm Superose 200 columns attached in series and eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 20 mM butylated hydroxytoluene (BHT), and 1 mM Trolox. Total cholesterol in the FPLC fractions was measured as above. SAA was measured using an ELISA prepared with monoclonal anti-mouse SAA (BioSource International). All mice were housed 4 per cage in autoclaved, filter-top cages with autoclaved water and kept on a 12-hour light/dark cycle. Animal care and use for all procedures was done in accordance with guidelines approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

BMT. BMT was performed as previously described (38). Briefly, BM-recipient animals received 10 Gy γ-irradiation followed by reconstitution of BM from $Tlr2^{-/-}$ or $Tlr2^{+/+}$ mice. All BMT mice were allowed to recover from irradiation and BMT for 4 weeks, after which they were fed the HFD. Documentation of successful reconstitution was established by PCR genotyping of recipient mouse spleen or femoral BM cells (Supplemental Figure 5). In the first BMT study, irradiated $Ldlr^{-/-}$ mice were reconstituted with marrow from wild-type $Tlr2^{+/+}$ or $Tlr2^{-/-}$ mice so that only BM-derived cells lacked expression of TLR2. Additionally, irradiated $Ldlr^{-/-}Tlr2^{-/-}$ double-mutant mice were reconstituted with marrow from $Tlr2^{-/-}$ or wild-type $Tlr2^{+/+}$ mice so that only BM-derived cells expressed TLR2. Animals were fed the HFD for 16 weeks.

In the second BMT study, irradiated $Ldlr^{-/-}$ mice were reconstituted with marrow from wild-type $Tlr2^{+/+}$ or $Tlr2^{-/-}$ mice. Two weeks after the transplantation, animals received an intravenous injection of 25 mg/kg body weight $GdCl_3$, which accelerated tissue macrophage turnover with the reconstituted BM-derived stem cells (39). At 4 weeks after BMT, animals were divided into groups receiving i.p. injections of vehicle or 50 μg of Pam3 (InvivoGen). Animals were fed the atherosclerotic diet for 12 weeks. Injections of Pam3 into $Ldlr^{-/-}$ and $Ldlr^{-/-}Tlr2^{-/-}$ mice were also conducted. In these studies, the animals were fed the HFD at 12–15 weeks of age, and they consumed the diet for 12 weeks.

Evaluation of atherosclerosis. Atherosclerotic lesion severity was assessed in the aortae as previously described (40). In brief, at euthanasia, animals were perfused with PBS, followed by formal-sucrose (4% paraformaldehyde and 5% sucrose in PBS, pH 7.4). The entire mouse aorta was dissected from the proximal ascending aorta to the bifurcation of the iliac artery by using a dissecting microscope. Adventitial fat was removed, and the aorta was opened longitudinally, pinned flat onto black dissecting wax, stained with Sudan IV, and photographed at a fixed magnification. The photographs were digitized, and total aortic areas and lesion areas were calculated by using Adobe Photoshop version 7.0 and NIH Scion Image software (http://rsb.info.nih.gov/nih-image/Default.html). The results were reported as a percentage of the total aortic area that contained lesions.

As a second assessment of atherosclerosis, lesions of the aortic root (aortic sinus) were analyzed by a modification of current methods of valve lesion analysis (40). Utilizing stereological principles, lesion volume was estimated across a fixed distance of the aortic sinus. Our preliminary data demonstrated that lesion volume estimation, compared with lesion cross-sectional area estimation, was a more conservative and robust method for estimating aortic sinus atherosclerosis in mice. After overnight fixation of hearts in 4% paraformaldehyde and 5% sucrose, they were cut at an angle approximately 100–120° (clockwise) from the long axis of the heart and embedded in OCT. Frozen hearts were sectioned on a Leica cryostat, with 14-μm sections collected from the beginning of the aortic sinus (defined as when a valve leaflet became visible) to 500 μm. For hearts cut at an angle that resulted in the valve leaflets not appearing in the same section (due to poor section angle), the lagging leaflet was used to determine the 500-μm distance. Due to the relatively stricter requirements of aortic lesion volume analysis, hearts cut at severe angles that were not suitable for analysis were excluded. Sections were collected in duplicate at 42-μm intervals. Sections were stained with oil red O, counterstained with Gill hematoxylin 1 (Fischer Scientific International), photographed, and digitized for lesion analysis.

Scoring of valve lesion areas was done for each of the 3 valve cusps individually. Lesion areas found only within the valve cusp were measured. Lesion volume estimation was determined from a 1 in 10 sampling rate; hence, valve cusps spaced at 140 μm were used to determine the lesion volume for a total of 4 sections analyzed per valve cusp. Lesion volume was calculated from an integration of the measured cross-sectional areas. Prediction of the coefficient of error (CE) in approximating lesion volume was computed using the Cavalieri estimator derived from a covariogram analysis of an ordered set of estimates of cross-sectional areas. This yielded

CE values of less than 10% and was acceptable for a stereological computation of the lesion volume.

Statistics. All scatter plots are expressed with the mean ± SD. Atherosclerosis data were either analyzed by the unpaired Student's *t* test (for normally distributed values, as common with aortic sinus lesion volume) or the nonparametric Mann-Whitney rank sum test (for non-normally distributed values, as common with aortic lesion area percentage). One-factor ANOVA or the nonparametric Kruskal-Wallis ANOVA on ranks was used to determine whether differences among multiple (greater than 2) groups existed with the treatment. For each data set, a normality test ($P > 0.05$) and an equal variance test ($P > 0.05$) were performed to identify normally distributed data. If either test failed, the nonparametric test was performed. Body weight and total plasma cholesterol changes over time were analyzed utilizing 2-factor repeated measures ANOVA. The analysis of factor level effects was done by the Holm-Sidak test of pairwise multiple comparisons. All statistical analysis was done with the use of the SigmaStat 3.00 (SPSS Inc.). A value of $P < 0.05$ was considered significant.

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