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Discoidin Domain Receptor 1 on Bone Marrow–Derived Cells Promotes Macrophage Accumulation During Atherogenesis

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Rationale: We described a critical role for the discoidin domain receptor (DDR1) collagen receptor tyrosine kinase during atherosclerotic plaque development. Systemic deletion of Ddr1 in Ldlr−/− mice accelerated matrix accumulation and reduced plaque size and macrophage content. However, whether these effects reflected an independent role for macrophage DDR1 during atherogenesis remained unresolved.

Methods: In the present study, we performed sex-mismatched bone marrow transplantation using Ddr1+/+;Ldlr−/− and Ddr1−/−;Ldlr−/− mice to investigate the role of macrophage DDR1 during atherogenesis. Chimeric mice with deficiency of DDR1 in bone marrow–derived cells (Ddr1−/−+/+) or control chimeric mice that received Ddr1+/+;Ldlr−/− marrow (Ddr1+/+−+/+) were fed an atherogenic diet for 12 weeks.

Results: We observed a 66% reduction in atherosclerosis in the descending aorta and a 44% reduction in plaque area in the aortic sinus in Ddr1−/−+/+ mice compared to Ddr1+/+−+/+ mice. Furthermore, we observed a specific reduction in the number of donor-derived macrophages in Ddr1−/−+/+ plaques, suggesting that bone marrow deficiency of DDR1 attenuated atherogenesis by limiting macrophage accumulation in the plaque. We have also demonstrated that the effects of DDR1 on macrophage infiltration and accumulation can occur at the earliest stage of atherogenesis, the formation of the fatty streak. Deficiency of DDR1 limited the appearance of 5-bromodeoxyuridine–labeled monocytes/macrophages in the fatty streak and resulted in reduced lesion size in Ldlr−/− mice fed a high fat diet for 2 weeks. In vitro studies to investigate the mechanisms involved revealed that macrophages from Ddr1−/− mice had decreased adhesion to type IV collagen and decreased chemotactic invasion of type IV collagen in response to monocyte chemoattractant protein-1.

Conclusions: Taken together, our data support an independent and critical role for DDR1 in macrophage accumulation at early and late stages of atherogenesis. (Circ Res. 2009;105:1141-1148.)

Key Words: atherosclerosis ■ discoidin domain receptor ■ collagen ■ macrophage ■ inflammation

The accumulation of macrophages in the arterial intima is a critical event in atherosclerotic plaque development. Macrophage accumulation depends on a series of well-defined interactions with the endothelium, culminating in transmigration and invasion of the subendothelial extracellular matrix. In the atherosclerotic intima, monocytes/macrophages interact with an extracellular matrix rich in several types of collagen. Collagens are important components of the extracellular matrix present within atherosclerotic plaques: contributing to lesion volume, enhancing the mechanical stability of the fibrous cap, and providing key signals that regulate monocyte differentiation, protease expression, and the production of inflammatory mediators.

The discoidin domain receptors (DDRs) are a subfamily of receptor tyrosine kinases that transduce signals when bound to collagens. There are 2 Ddr genes in the human and mouse genomes, Ddr1 and Ddr2, and 6 differentially spliced isoforms of Ddr1 have been identified (termed Ddr1a-e, and an isoform only expressed in rat testes). Both DDR1 and DDR2 bind to several collagen subtypes, but the receptors require an intact triple helical domain for signaling; denatured collagen, or gelatin, does not induce signaling through DDRs. Importantly, DDR1 has been shown to signal when bound to collagen types I to V and VIII, a ligand repertoire that includes fibril forming interstitial collagens (types I to III), as well as network-forming type IV collagen, a principal component of the endothelial basement membrane.

Ferri et al have reported the expression of DDR1 in the atherosclerotic plaques of nonhuman primates. We have recently identified a functional role for DDR1 in the regula-
tion of atherosclerotic plaque inflammation and fibrosis using \textit{Ldlr}⁻/⁻ mice fed a high-fat diet.\textsuperscript{10} Deletion of DDR1 resulted in smaller atherosclerotic plaques that were matrix rich and macrophage poor, both key features of a stable plaque. Accelerated matrix accumulation in \textit{Ddr1}⁻/⁻;\textit{Ldlr}⁻/⁻ plaques resulted from enhanced expression of collagens and elastin by \textit{Ddr1}⁻/⁻ smooth muscle cells (SMCs) and was associated with decreased in situ matrix metalloproteinase (MMP) activity, as well as reduced lesional macrophage content. However, because DDR1 is expressed both on vessel wall–derived cells such as SMCs and on bone marrow–derived cells such as macrophages, the relative contribution of DDR1 expressed solely by macrophages to plaque development remained unresolved.

In the present study, we have used sex-mismatched bone marrow transplantation to study the role of DDR1 expressed on bone marrow–derived cells such as monocytes/macrophages during atherogenesis. Atherosclerotic plaques from chimeric mice with DDR1-deficient bone marrow (\textit{Ddr1}⁻/⁻;\textit{Ldlr}+/⁺/⁺) were smaller in size and exhibited reduced accumulation of bone marrow–derived macrophages. Bromodeoxyuridine (BrdUrd) pulse-labeling experiments performed in \textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ and \textit{Ddr1}⁻/⁻;\textit{Ldlr}⁻/⁻ mice fed an atherogenic diet for 2 weeks revealed a novel role for DDR1 in macrophage accumulation in the early fatty streak. The mechanisms behind this were revealed by in vitro experiments showing that macrophages from \textit{Ddr1}⁻/⁻;\textit{Ldlr}⁻/⁻ mice exhibited decreased attachment to and invasion of type IV collagen matrix. Our data support an independent role for macrophage DDR1 in atherogenesis and suggest that DDR1 promotes macrophage accumulation and lesion growth by regulating invasion of the intimal basement membrane.

Methods
An expanded Methods section can be found in the Online Data Supplement at http://circres.ahajournals.org.

Reciprocal Bone Marrow Transplantation, Analysis of Atherosclerosis, and Histomorphometry
Animal experiments were performed in accordance with the guidelines of the Canada Council on Animal Care. Sex-mismatched bone marrow transplantation was performed using \textit{Ddr1}⁻/⁻;\textit{Ldlr}⁻/⁻ mice and their \textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ littermates.\textsuperscript{10} The experimental groups included female \textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ hosts receiving male \textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ bone marrow (\textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ control), and female \textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ hosts receiving male \textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ bone marrow (\textit{Ddr1}⁻/⁻;\textit{Ldlr}⁻/⁻ bone marrow donor). Three weeks after transplantation, mice were placed on an atherogenic diet containing 40% kcal of fat and 1.25% cholesterol by weight (Research Diets, D12108) for 12 weeks. After 12 weeks, mice were euthanized by anesthetic overdose and the heart, kidneys, and descending aorta (downstream of the left subclavian artery to the iliac bifurcation) were fixed in 4% paraformaldehyde. The aortic sinus was embedded in paraffin and sectioned into 5-μm-thick cross-sections. Details of the methods used for analysis of male-female chimerism can be found in the Online Data Supplement. Plasma lipid analysis, oil red O staining of the descending aorta, Verhoeﬀ’s van Gieson, picosirius red and LC-PolScope analysis of fibrillar collagen were performed as described previously, and additional details can be found in the online supplement.\textsuperscript{10}

\textbf{Y Chromosome Fluorescence In Situ Hybridization and Analysis of Plaque Cellular Content}
After deparaffinization in xylenes, air-dried cross-sections of the aortic sinus were denatured in 10 mmol/L sodium citrate (pH 6.0) for 2 hours at 80°C, rinsed in 2× sodium chloride/sodium citrate buffer (SSC), and digested in Pepsin (0.22 mg/mL) in 0.1 mol/L HCl for 2 hours at 37°C. Dehydrated sections were then incubated with 3.3 μL of CY3-labeled mouse Y chromosome Paint probe (Cambio), coverslipped, and sealed with rubber cement before overnight hybridization at 37°C. After posthybridization washes in 0.1× SSC/0.3% NP-40 at 75°C for 3 minutes and 4× SSC/0.1% NP-40 for 10 minutes, slides were counterstained with Hoechst 33528 (1:10 000 in distilled water). Y chromosome–positive cells were counted using a Zeiss Axioplan epifluorescence microscope with a ×100 oil immersion lens.

To determine the relative contribution of vessel wall (ie, host) and bone marrow (ie, donor)–derived cells to lesion, the number of Y chromosome–positive cells was subtracted from the total number of nuclei in the plaque. The difference reflects the number of resident vessel wall–derived cells, for example SMCs, in the lesion.

\textbf{BrdUrd Pulse Labeling and Measurement of Fatty Streak Lesion Size}
Macrophage accumulation into the fatty streak was assessed by BrdUrd pulse labeling.\textsuperscript{11,12} \textit{Ddr1}⁺/⁺;\textit{Ldlr}⁻/⁻ and \textit{Ddr1}⁻/⁻;\textit{Ldlr}⁻/⁻ mice were fed the atherogenic diet for 2 weeks, then given a single IV injection of BrdUrd (10 mg/mL, 0.2 mL volume) to label a cohort of proliferating cells in both the bone marrow and aortic intima at the time of the injection. Mice were euthanized 2 or 24 hours later, and the ascending aorta (aortic sinus to brachiocephalic artery) was whole mount immunostained for BrdUrd using fluorescein isothiocyanate (FITC)-tyramide amplification (Tyramide signal amplification; Perkin Elmer) and mounted en face. Costaining of nuclei and lipids was performed using Hoechst 33528 (1:10 000 in distilled H2O) and oil red O, respectively.

The total number of BrdUrd-positive cells in the ascending aorta was counted using an Olympus Fluoview confocal microscope with a ×60 oil immersion lens. As previously reported, BrdUrd-positive cells cannot be detected in the circulation at 2 hours\textsuperscript{11}; therefore, the number of BrdUrd-positive cells in the aortic intima at 2 hours provides a baseline measurement of intimal macrophage proliferation. In contrast, between 6 and 24 hours, there is a detectable cohort of BrdUrd-positive monocytes present in the circulation.\textsuperscript{11} Therefore, an increase in the BrdUrd-positive cell number in the aortic intima between 6 and 24 hours reflects the accumulation of newly labeled monocytes/macrophages in the developing fatty streak by a combination of local proliferation and recruitment from the circulation over a 22 hour time period. The number of BrdUrd-positive cells per ascending aorta at each time point is expressed per unit of fatty streak lesion surface area to account for differences in lesion size between groups.

Measurement of fatty streak lesion size was carried out using oil red O fluorescence and confocal microscopy as detailed in the online supplement. Low magnification images of the en face preparations of ascending aorta were captured using a Bio-Rad confocal microscope with a ×4 objective. Measurement of the percentage of aortic surface
Chimerism, and Plasma Lipids

Ddr1 weeks on the atherogenic diet was assessed by analyzing (Online Table I). Male:female chimerism measured after 12 weeks on the atherogenic diet (15 weeks Body weight and fasting plasma cholesterol and triglycerides analyzed by Mann–Whitney using Student's comparisons between transplant groups or genotypes were performed Data are presented as means SEM. All statistical analysis was significance was determined at

Flow Cytometry

Ddr1+/−:Ldlr−/− or Ddr1−/−:Ldlr−/− mice fed the atherogenic diet for 2 weeks were pulse labeled with BrdUrd for 24 hours, and samples of peripheral blood (100 μL) or bone marrow (10⁶ cells) were taken to measure intracellular staining of BrdUrd using flow cytometry (FITC-BrdUrd flow kit, Becton Dickson) according to the instructions of the manufacturer. Costaining for monocytes in peripheral blood was performed using the following markers: CD115-phycoerythrin (1:100, eBioscience), Ly-6C-biotin (1:200, BMA), and streptavidin-allophycocyanin (SA-APC, 1:800, Becton-Dickinson). Samples were analyzed on a Beckman Coulter FC500 flow cytometer at the following wavelengths: FITC: 510 nm/540 nm, phycoerythrin: 560 nm/590 nm; APC: 660 nm/690 nm.

Statistical Analysis

Data are presented as means±SEM. All statistical analysis was carried out using Sigma Stat (SyStat Software Inc). Pairwise comparisons between transplant groups or genotypes were performed using Student’s t test. Data that did not fit a normal distribution were analyzed by Mann–Whitney U test for nonparametric comparisons. BrdUrd pulse data were analyzed using a 1-way ANOVA with a Tukey post hoc analysis for pairwise comparisons. Statistical significance was determined at P<0.05.

Results

Bone Marrow Transplantation, Assessment of Sry Chimerism, and Plasma Lipids

Body weight and fasting plasma cholesterol and triglycerides measured after 12 weeks on the atherogenic diet (15 weeks after transplant) were comparable between transplant groups (Online Table I). Male:female chimerism measured after 12 weeks on the atherogenic diet was assessed by analyzing genomic DNA isolated from peripheral blood leukocytes for Ddr1 and Sry, a marker of the Y chromosome (Online Figure I, A). Leukocyte DNA from Ddr1+/−+/− mice demonstrated the presence of the donor genotype (Ddr1−/−), with negligible host DNA remaining. (Online Figure I, A, top). The abundance of leukocyte Sry was comparable in all chimeric mice (Online Figure I, A, bottom), and this was confirmed by measuring the ratio of Sry/Gapdh by quantitative real-time PCR (Online Figure I, B).

DDR1 Expressed on Bone Marrow–Derived Cells Promotes Atherogenesis

Deletion of Ddr1 in bone marrow–derived cells (Ddr1−/−+/+) resulted in a marked reduction in atherosclerotic plaque burden in the descending aorta compared to Ddr1+/−+/+ mice (Figure 1A). Quantification of the percentage aortic surface area occupied by oil red O–positive plaque revealed a significant 66% decrease in atherosclerotic plaque burden in Ddr1−/−+/+ mice compared to Ddr1+/−+/+ mice (Figure 1B). Furthermore, atherosclerotic plaque area measured in cross-sections of the aortic sinus was reduced by 44% in Ddr1−/−+/+ mice compared to Ddr1+/−+/+ mice (Figure 1C). Taken together, these data confirm a critical role for DDR1 expressed on bone marrow–derived cells in atherosclerotic plaque growth and development.

Bone Marrow–Derived Macrophage Accumulation Was Reduced in Ddr1−/−+/+ Plaques

Because male bone marrow donors were used to generate the chimeric mice, we were able to detect bone marrow–derived cells in the lesions using fluorescence in situ hybridization for the Y chromosome. Y chromosome–positive cells were abundant within the plaque and were also observed in the adventitia but were not observed in the media (Figure 2A and 2B). Correlative immunofluorescence staining with antibodies against Mac-2, smooth muscle α-actin, or vascular cell adhesion molecule-1 confirmed that the majority of the lesions Y chromosome–positive cells were macrophages (Online Figure II and data not shown). Quantification of the number of Y chromosome–positive cells in the plaques revealed a significant 47% decrease in bone marrow–derived macrophage accumulation in Ddr1−/−+/+ mice compared to Ddr1+/−+/+ mice (Figure 2C, gray bars). Furthermore, the decrease in bone marrow–derived macrophages was responsible for the decrease in total cell number that was observed in the aortic sinus lesions of Ddr1−/−+/+ mice.
Compared to Ddr1+/+→+/+ mice (467±53 versus 324±24; Figure 2C, sum of black and gray bars). In contrast, bone marrow–specific deletion of DDR1 had no effect on the number of host-derived resident vessel wall cells (Figure 2C, black bars). Taken together with the reduction in lesion size, these data suggest that deficiency of DDR1 in bone marrow–derived cells specifically limits the accumulation of macrophages in the developing plaques, which results in the attenuation of lesion growth.

The net accumulation of macrophages in the atherosclerotic plaque is influenced by macrophage infiltration into the lesion and cell death within the lesion. To determine whether the reduction in macrophage number in Ddr1−/−→+/+ plaques was caused by an increase in cell death, we measured the percentage of apoptotic cells in sections of the aortic sinus using TUNEL. The percentage of TUNEL-labeled cells was significantly decreased in Ddr1−/−→+/+ mice compared to Ddr1+/+→+/+ mice (Figure 2D). Therefore, these data are consistent with the hypothesis that the reduction in plaque macrophages observed in Ddr1−/−→+/+ mice was the result of impaired infiltration into the lesion and not attributable to increased cell death.

Matrix Composition Was Similar in Ddr1−/−→+/+ and Ddr1+/+→+/+ Plaques

To evaluate changes in matrix composition of the atherosclerotic plaques from Ddr1+/+→+/+ and Ddr1−/−→+/+ mice, we stained serial sections of aortic sinus with Verhoeff’s van Gieson and picrosirius red to identify elastin and collagen respectively (Figure 3). Despite the significant reduction in plaque size, measurement of the percentage of lesion area occupied by collagen or elastin demonstrated no difference between groups. Similarly, using the LC-PolScope, which detects the retardance of polarized light by fibrillar collagens, we observed comparable fibrillar collagen content of plaques between transplant groups (Figure 3).

Deficiency of DDR1 Limits the Infiltration of BrdUrd-Labeled Monocytes/Macrophages in the Developing Fatty Streak

Because our findings on well-developed atherosclerotic plaques indicated reduced macrophage accumulation in the Ddr1−/−→+/+ lesions that could be attributed to decreased macrophage infiltration, we wished to determine whether this deficit was also present at the earliest stages of lesion development: the formation of the fatty streak. The fatty streak provides a simplified in vivo model to study the role of DDR1 in macrophage infiltration because the majority of lesional cells are macrophage foam cells and there is little SMC involvement or lesion fibrosis. We performed BrdUrd pulse labeling in Ddr1+/+;Ldlr−/− and Ddr1−/−;Ldlr−/− mice fed an atherogenic diet for 2 weeks. Mice were given a single pulse injection of BrdUrd, which labeled a cohort of proliferating cells in the bone marrow and allowed us to track their subsequent infiltration and accumulation in the atherosclerotic intima using en face confocal microscopy.

Fasting plasma lipids assayed after 2 weeks on the atherogenic diet were comparable between the two genotypes (Online Table II), and the proportion of BrdUrd-positive monocytes in the circulation and the bone marrow were also similar between groups (Figure 4A and 4B). Measurement of the percentage of ascending aortic surface area occupied by fatty streak was carried out by en face confocal imaging, which revealed a significant decrease in lesion size in Ddr1−/−;Ldlr−/− mice compared to Ddr1+/+;Ldlr−/− mice (Figure 4C). These data provide the first evidence of a role for DDR1 as a regulator of fatty streak formation. Independent of the change in lesion size, the deficiency of DDR1 also limited the infiltration of BrdUrd labeled monocytes/macrophages into the fatty streak. Figure 5A and 5B show the extent of oil red O–stained fatty streak, and Figure 5C and 5D show the BrdUrd-labeled cells in the lesions. Ddr1+/+;Ldlr−/− mice exhibited a significant increase in the number of BrdUrd+ cells compared to Ddr1−/−→+/+ mice (600±1144 versus 324±24; Figure 2C, sum of black and gray bars). In contrast, bone marrow–specific deletion of DDR1 had no effect on the number of host-derived resident vessel wall cells (Figure 2C, black bars). Taken together with the reduction in lesion size, these data suggest that deficiency of DDR1 in bone marrow–derived cells specifically limits the accumulation of macrophages in the developing plaques, which results in the attenuation of lesion growth.

(matrix composition was similar in Ddr1−/−→+/+ and Ddr1+/+→+/+ plaques)

(deficiency of DDR1 limits the infiltration of BrdUrd-labeled monocytes/macrophages in the developing fatty streak)
cells per unit of lesion area between 2 and 24 hours (Figure 5E), indicating that an accumulation of labeled macrophages occurred during this time period. By contrast, the number of BrdUrd\(^{+}\) cells per unit of lesion area in the Ddr1\(^{-/-}\)::Ldlr\(^{-/-}\) mice was unchanged between 2 and 24 hours (Figure 5E). Taken together, these data suggest that macrophage infiltration in the fatty streak was impaired in Ddr1\(^{-/-}\)::Ldlr\(^{-/-}\) mice, which resulted in reduced fatty streak lesion size.

To determine whether the attenuation of macrophage infiltration in DDR1-deficient animals also occurred in response to a general inflammatory stimulus, we measured macrophage accumulation in the peritoneum after an injection of thioglycollate. The percentage of macrophages in the peritoneal lavage was 12.3\(\pm\)2.3\% in Ddr1\(^{-/-}\) mice compared to 15.5\(\pm\)1\% in Ddr1\(^{+/+}\) mice; however, the difference was not statistically significant.

### Discussion

Using a bone marrow transplantation approach, we have identified an independent role for DDR1 expressed on macrophages in contributing to atherosclerotic plaque development by regulating macrophage infiltration and accumulation in the plaque. The reduction in lesion size and cellularity in

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**Figure 3.** Similar matrix composition of atherosclerotic plaques from Ddr1\(^{-/-}\) mice and Ddr1\(^{+/+}\) mice. A, B, D, and E, Light microscopic images (A and B) and corresponding PolScope images (D and E) of picrosirius red stained cross-sections from the aortic sinus of chimeric mice to detect collagen. C and F, Quantification of the percentage plaque area positive for picrosirius red staining on light microscopic images (C) or the average birefringence retardance of fibrillar collagen measured by LC-PolScope (F). G and H, Light microscopic images (A and B) and corresponding PolScope images (D and E) of picrosirius red stained cross-sections from the aortic sinus from chimeric mice stained with Verhoeff’s van Gieson to detect elastin. I, Quantification of the percentage of plaque area that stained positive for elastin (n=7 animals per group). Scale bar=100 \(\mu\)m.

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**DDR1 Regulates Macrophage Infiltration by Promoting the Interaction With and Invasion of Type IV Collagen Matrices**

Emerging evidence supports a role for DDR1 as a mediator of matrix invasion in multiple cell types.\(^2\) Therefore, to further investigate the mechanisms behind the reduction in macrophage accumulation, we assayed the adhesion and invasion of peritoneal macrophages from Ddr1\(^{+/+}\)::Ldlr\(^{-/-}\) and Ddr1\(^{-/-}\)::Ldlr\(^{-/-}\) mice through type IV collagen matrices in vitro. Type IV collagen is a principal component of the endothelial basement membrane and likely the first collagenous barrier to be encountered by a macrophage invading the plaque. Adhesion of Ddr1\(^{-/-}\) macrophages to type IV collagen was significantly reduced by 51\% compared to Ddr1\(^{+/+}\) cells (Figure 6A). Next, we assayed the monocyte chemoattractant protein-1–dependant chemotaxis of Ddr1\(^{+/+}\) or Ddr1\(^{-/-}\) peritoneal macrophages through type IV collagen and found that Ddr1\(^{-/-}\) macrophages exhibited a 50\% reduction in chemotactic invasion through type IV collagen compared to Ddr1\(^{+/+}\) macrophages (Figure 6B). These data suggest that deficiency of DDR1 limits the ability of macrophages to adhere to and invade through collagen matrix barriers, key steps in their infiltration and accumulation in the arterial intima.
Ddr1−/−;Ldlr−/− mice was attributable to decreased bone marrow–derived macrophage accumulation and was associated with impaired adhesion and invasion through type IV collagen matrices by Ddr1−/− macrophages. Furthermore, we have determined that DDR1-dependant macrophage accumulation is also critical for the formation of the fatty streak, one of the earliest stages of lesion development. Taken together, our data suggest that DDR1 expression on macrophages, by facilitating the interaction with and invasion through type IV collagen–rich matrices such as the endothelial basement membrane, mediates macrophage accumulation and contributes to lesion growth at multiple stages.

We show that deletion of DDR1 on bone marrow–derived macrophages is sufficient to result in decreased plaque growth at both early (2 weeks) and late (12 weeks) stages of plaque development. That the reduction in plaque size is a consequence of reduced macrophage accumulation is evidenced by comparing the specific decrease in donor-derived macrophage cell number (47% decrease), and the parallel and equivalent reduction in plaque size (44% decrease). Furthermore, even at the earliest stages of plaque development, deficiency of DDR1 limits the infiltration of BrdUrd-labeled monocytes/macrophages into the lesion. By allowing us to dissect out the relative role of DDR1 on macrophages versus SMCs in plaque growth, these novel findings expand on our previous work examining the effects of systemic deletion of DDR1 during atherogenesis10 and point to an important and independent role for DDR1 on macrophage infiltration to the atherosclerotic plaque.
matrix invasion. Furthermore, collagen signaling has been shown to regulate the production of MMP-1 and MMP-9 in human macrophages. These data strongly suggest that the regulation of MMP expression is the mechanism for DDR1-mediated matrix invasion. Taken together, these findings suggest that DDR1 deficiency in macrophages limits macrophage invasion of the endothelial basement membrane and plaque extracellular matrix.

To assess whether DDR1 deficiency also limited macrophage infiltration to a more general inflammatory stimulus, we assayed peritoneal macrophage accumulation after an injection of thioglycollate. We observed a trend toward a reduction in peritoneal macrophage accumulation in DDR1-deficient mice; however, the reduction was not statistically significant and was not as great as we observed in the atherosclerotic plaque. Recently, Voisin et al demonstrated that monocytes preferentially traverse the basement membrane of postcapillary venules at sites of low matrix protein expression, termed low expression regions (LERs). Moreover, their data suggest that monocyte transmigration at LERs does not require proteases. Because LERs have not been demonstrated in the matrix rich subendothelial basement membrane of the aortic intima, it is possible that protease dependant transmigration at this arterial location could account for the differences observed between macrophage infiltration in atherosclerotic plaques versus peritoneal inflammation.

Despite the dramatic change in plaque size and cell accumulation, the atherosclerotic plaques from Ddr1<sup>-/-</sup>:Ldlr<sup>−/−</sup> chimeric mice exhibited no difference in the proportion of lesion area occupied by matrix molecules compared to Ddr1<sup>+/−</sup>:Ldlr<sup>−/−</sup> controls. By contrast, in previous work examining the advanced plaques of Ddr1<sup>-/-</sup>:Ldlr<sup>−/−</sup> mice (with systemic deletion of DDR1), we observed an acceleration of collagen and elastin deposition and increased fibrosis compared to Ddr1<sup>+/−</sup>:Ldlr<sup>−/−</sup> mice, and we also documented increased collagen and elastin expression by DDR1-deficient SMCs in vitro. This suggests that enhanced matrix accumulation is the result of DDR1 deletion in SMCs alone, and that macrophage DDR1 deficiency does not contribute to the enhanced matrix accumulation observed in the lesions of Ddr1<sup>-/-</sup>:Ldlr<sup>−/−</sup> mice.

Emerging evidence supports a role for DDR1 in leukocyte accumulation in chronic inflammatory diseases such as renal and pulmonary fibrosis. Using bone marrow transplantation, we demonstrate, for the first time, an independent role for macrophage DDR1 in atherosclerotic plaque development. Our data support a model whereby DDR1 facilitates the invasion and accumulation of monocytes/macrophages in the arterial intima by mediating the interaction with and invasion through the type IV collagen–rich endothelial basement membrane, resulting in reduced macrophage accumulation, decreased intimal inflammation, and the attenuation of atherogenesis.

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Disclosures

None.

References

Reciprocal bone marrow transplantation and analysis of atherosclerosis.

Animal experiments were performed in accordance with the guidelines of the Canada Council on Animal Care. Sex mismatched bone marrow transplantation was performed using Ddr1−/−;Ldlr−/− mice and their Ddr1+/+;Ldlr−/− littermates. Female Ddr1+/+;Ldlr−/− mice were lethally irradiated (9.5 Gy, Cs-137 source), and reconstituted after 4 hours via tail vein injection with 10 million bone marrow cells isolated from the femurs and tibias of male donor mice. Transplanted mice were housed in sterile cages with sterile food, bedding and water. The experimental groups included female Ddr1+/+;Ldlr−/− hosts receiving male Ddr1+/+;Ldlr−/− bone marrow (Ddr1+/+ → +/+; control), and female Ddr1+/+;Ldlr−/− hosts receiving male Ddr1−/−;Ldlr−/− bone marrow (Ddr1−/− → +/+; bone marrow deletion).

Three weeks after transplantation mice were placed on an atherogenic diet containing 40% K cal fat and 1.25 % cholesterol by weight (Research Diets, D12108) for 12 weeks. After 12 weeks, mice were sacrificed by anesthetic overdose with 67 mg/kg xylazine and 333 mg/kg ketamine i.p. and blood samples were collected for analysis of plasma lipids as previously described. The left ventricle was cannulated and tissues were perfused with 50 ml of sterile saline. The heart, kidneys and descending aorta (downstream of the left subclavian artery to the iliac bifurcation) were fixed in 4 % paraformaldehyde. The descending aorta was used for enface analysis of atherosclerotic plaque burden by oil red o staining as previously described. The aortic sinus was embedded in paraffin and sectioned into 5 um thick cross-sections. Plaque area was measured in V erhof’s van gieson (VVG) stained cross-sections sections using digital image analysis software (Simple PCI). Atherosclerotic plaque present on the aortic valve leaflets was excluded from this analysis.

Assessment of male:female chimerism.

To assess the efficiency of bone marrow reconstitution, genomic DNA was isolated from peripheral blood leukocytes of chimeric mice and quantified by UV absorbance at 260 nm. 45 ng of total DNA was assayed by PCR amplification for Ddr1 genotype as previously described and using primers for mouse Sry, a marker of the Y chromosome (forward: 5’-CGTGGTTGAGAGGCACAAGT-3’ and reverse: 5’-AACAGGCTGCCAATAAACG-3’). PCR reactions for Sry were performed as follows: after 2 minutes at 94 °C, the reaction was cycled 35 times at 94°C for 45 seconds, 58 °C for 45 seconds and at 72 °C for 1 minute. Male:female chimerism was also assessed by real time PCR using primers for Sry and Gapdh. Power SY BR Green PCR Master Mix (Applied Biosystems) was used to amplify 9 ng of leukocyte DNA using the 7900HT sequence detection system (Applied Biosystems). Primer sequences used to amplify Sry were: forward: 5’-TTATGGTTGGTCCCGTGTTG-3’ and reverse: 5’-GGCCTTTTTCGGCTTCTGT-3’. Primers for Gapdh were: forward: 5’-AAGAGAGGCCCTATCCCAAATTCGAGC-3’ and reverse: 5’-CCTAGGCCCCCTGTTATTA-3’. After 10 minutes at 95 °C, the reactions were cycled 40 times at 95 °C for 15 seconds, and then 60 °C for 1 minute. Relative quantification of the ratio of Sry/Gapdh in each sample was performed using the formula 2−ΔΔCT and was expressed as a percent relative to the Ddr1+/+ transplant group.

Immunohistochemistry and matrix staining.
Cross sections of the aortic sinus were deparaffinized in xylenes, and rehydrated in an ethanol series. Sections were stained using antibodies against SM α−actin (1:400, Sigma), Mac-2 (1:100, Cedarlane). Sections were incubated with species appropriate biotinylated secondary antibodies followed by incubation with avidin-biotin-HRP complex (ABC elite, Vector Laboratories). Sections were incubated with the chromogenic substrate 3,3’-diaminobenzidine (Dako Corp), followed by counterstaining with hematoxylin (1:50). For immunofluorescent detection of Mac-2 or SM α−actin, species appropriate, Cy3-conjugated secondary antibodies were used and nuclei were visualized with Hoechst 33528 (1:10000 in dH2O). Negative controls were performed by omitting the primary antibody. Additional serial sections were stained with picrosirius red (PSR) or Verhoff’s van geison (VVG) for collagen or elastin, respectively. Fibrillar collagen content of lesions was assessed using the LC Polscope, as previously described.1 The percentage of positively stained plaque area was assessed by colour thresholding of maximum and minimum signals (Simple PCI, C-1 Imaging).

Y chromosome fluorescence in situ hybridization (FISH) and analysis of plaque cellular content.

Cross sections of the aortic sinus were deparaffinized in xylenes, washed in ethanol and air dried, before incubation in 10mM sodium citrate pH 6.0 for 2 hours at 80°C. Sections were then rinsed in 2X sodium chloride-sodium citrate buffer (SSC) then digested in Pepsin (0.22 mg/ml) in 0.1M HCl for 2 hours at 37°C. After washing in distilled water, sections were dehydrated through an ethanol series and incubated with 3.3 ul of CY3 labelled mouse Y chromosome Paint probe (Cambio), according to the manufacturer’s instructions. Sections were coverslipped with 12mm round coverslips and sealed with rubber cement. Sections and probe were denatured at 78 °C for 10 minutes, followed by hybridization for 22 hours in a humidified chamber at 37 °C. After hybridization, the rubber cement was removed and slides were washed in 0.1X SSC/0.3% NP-40 at 75°C for 3 minutes followed by a wash in 4X SSC/0.1% NP-40 for 10 minutes. Slides were then rinsed in 1X SSC and sections were counter-stained with Hoechst 33528 (diluted 1:10000 in distilled water) for 10 minutes, washed in distilled water and coverslipped with Prolong Gold Antifade solution (Molecular Probes). Y chromosome positive cells were counted using a Zeiss Axiopt epifluorescence microscope with a 100X oil immersion lens. Representative images of FISH positive cells were obtained using an Olympus Fluoview confocal microscope with a 60X objective and 2.2X zoom at the following excitation/emission wavelengths: CY3: 543 nm excitation / 567 nm emission, Hoechst: 405nm excitation / 461 nm emission. A DIC image overlay was also captured to allow visualization of tissue morphology.

Analysis of the cellular composition of the lesions was carried out using cell counts. To determine the relative contribution of vessel wall (i.e. host) and bone marrow (i.e. donor) derived cells to lesion, the number of Y chromosome positive cells was subtracted from the total number of nuclei in the plaque. The difference reflects the number of resident vessel wall derived cells, for example SMCs, in the lesion.

TUNEL Staining.

Apoptosis in atherosclerotic plaques was detected by the Terminal deoxynucleotide transferase (TdT) mediated dUTP nick end labeling (TUNEL) method using the Apoptag peroxidase kit (Chemicon). Briefly, sections of the aortic sinus from chimeric mice were deparaffinized in xylenes, rehydrated through an ethanol series and digested in 20 ug/ml proteinase K for 1 hour at room temperature. After blocking endogenous peroxidase activity with 3% H2O2, sections were incubated with 10 ul of TdT enzyme and reaction buffer solution, coverslipped with 12 mm round coverslips and incubated for 1 hour at 37°C. The TdT reaction adds digoxigenin-labeled dUTP nucleotides to the DNA strand breaks that are associated with
apoptotic cell death. Digoxigenin is a plant protein not present in mammalian cells, limiting the possibility of false positive staining due to antibody cross reactivity. After stopping the reaction in a stop/wash buffer, sections were incubated with peroxidase conjugated-anti digoxigenin antibody. Sections were then incubated with the chromogenic substrate 3,3′-diaminobenzidine (DAKO Corp), followed by counterstaining with hematoxylin (1:40). Negative controls were performed by omitting the TdT enzyme on serial sections. Positive controls were performed by incubating serial sections with 1U DNase I (0.1U/ul, Fermentas) to cause DNA strand breaks. The number of TUNEL positive nuclei in the lesions and total nuclei counts were counted at 40X by light microscopy. Data are expressed as the percentage of TUNEL positive nuclei per section.

**BrdU pulse labeling and measurement of fatty streak lesion size.**

Macrophage accumulation into the fatty streak was assessed by BrdU pulse labeling. Starting at 10-12 weeks of age, DDR1+/+;Ldlr-/ and DDR1-/-;Ldlr-/ mice were fed the atherogenic diet for 2 weeks, then given a single i.v. injection of BrdU (10 mg/ml, 0.2 ml volume) a thymidine analog with a short half life that is incorporated into newly synthesized DNA. The BrdU pulse results in the labeling of a cohort of proliferating cells in both the bone marrow and aortic intima at the time of the injection. Mice were sacrificed 2 or 24 hours later, by anesthetic overdose with 67 mg/kg xylazine and 333 mg/kg ketamine i.p. The vasculature was perfused at 100 mmHg with chilled saline followed by chilled 4% paraformaldehyde for 5 minutes. The aortic arch and heart were immersion fixed in 4% paraformaldehyde on ice for 1 hour, cleared of fat and adventitia, and the ascending aorta from the aortic sinus and including ostia of the brachiocephalic artery was isolated.

En face preparations of the ascending aorta were stained for BrdU. Vessels were washed in 0.1% Triton X-100 and 1% fetal bovine serum in PBS, then permeabilized in 10% DM SO/0.5% Triton x-100 for 30 minutes on an orbital shaker. After blocking endogenous peroxidase activity with 0.3% H2O2 for 30 minutes, vessels were treated with 555U DNase I (Invitrogen) for 1.5 hours at 37 °C and incubated with a biotinylated anti-BrdU monoclonal antibody (1:200 MD5215, Invitrogen) overnight at 4°C. The next day, vessels were washed and incubated with streptavidin-HRP (1:300) for 1 hour at room temperature followed by incubation with FITC-tyramide (1:50) according the manufacturer’s instructions (Tyramide signal amplification (TSA), Perkin Elmer). Counter staining of nuclei and lipids was performed using Hoescht 33528 (1:10000 in distilled H2O) and Oil red O, respectively.

The total number of BrdU positive cells in the ascending aorta was counted using an Olympus Fluoview confocal microscope with a 60X oil immersion lens. As previously reported, BrdU positive cells cannot be detected in the circulation at 2 hours, therefore the number of BrdU positive cells in the aortic intima at 2 hours provides a baseline measurement of intimal macrophage proliferation. In contrast, between 6 and 24 hours, there is a detectable cohort of BrdU positive monocytes present in the circulation (reference 10). Therefore, an increase in the BrdU positive cell number in the aortic intima between 2 and 24 hours reflects the appearance of newly labeled monocyte/macrophages in the developing fatty streak by a combination of local proliferation and recruitment from the circulation over a 22 hour time period. The number of BrdU positive cells per ascending aorta at each time point was expressed per unit of fatty streak lesion surface area to account for differences in lesion size between groups.

Since the lipid levels in these early plaques were too low to allow measurement of atherosclerotic plaque area by light microscopy, but Oil red O does fluoresce brightly, we exploited the increased sensitivity of Oil red O fluorescence to quantify fatty streak lesion size in these mice. Low magnification images of the en face preparations of ascending aorta were captured using a Biorad confocal microscope with a 4X objective. Images were captured at 543
nm excitation / 619 nm emission to detect the Oil red O stained lipid and 488 nm excitation / 519 nm emission to detect the autofluorescence of the internal elastic lamina. Measurement of the percentage of aortic surface area occupied by plaque was carried out using Simple PCI quantitative imaging software.

**Flow Cytometry.**

Ddr1+/+;Ldlr−/− or Ddr1−/−;Ldlr−/− mice fed the atherogenic diet for 2 weeks were pulse labeled with BrdU for 24 hours, and samples of peripheral blood (100 ul) or bone marrow (10⁶ cells) were taken to measure intracellular staining of BrdU using a flow cytometry (FITC-BrdU flow kit, Becton Dickinson) according to the manufacturer’s instructions. Co-staining for monocytes in peripheral blood was performed using the following markers: CD115-phycoerythrin (PE, 1:100, eBioscience), Ly6C-biotin (1:200, BMA) and Streptavidin-allophycocyanin (SA-APC, 1:800, Becton-Dickinson). Samples were analyzed on a Beckman Coulter FC500 flow cytometer at the following wavelengths: FITC: 510 nm – 540 nm, PE: 560 nm – 590 nm, APC: 660 nm – 690 nm.

**Peritoneal Macrophage Infiltration Assay.**

In order to investigate macrophage infiltration into the peritoneum, Ddr1+/+;Ldlr−/− and Ddr1−/−;Ldlr−/− mice (n=8), were injected with 1 ml of 4% brewers modified thioglycollate medium (BD) in the peritoneal cavity, and 24 hours later were subjected to a peritoneal lavage with 10 ml RPMI. From the total lavage, aliquots of 10⁶ cells in 1% BSA in RPMI were then incubated with unlabeled mAb or directly conjugated primary mAb for 30 min on ice. Staining was carried out using the following markers: CD115-phycoerythrin and Ly6C-biotin. Concentrations were the same as described above. Samples were analyzed on a Beckman Coulter FC500 flow cytometer as described above. Cells that were CD115+/LY6C+ were considered to be macrophages.

**Macrophage adhesion assay.**

Peritoneal macrophages were harvested from Ddr1+/+;Ldlr−/− or Ddr1−/−;Ldlr−/− mice by peritoneal lavage with cold RPMI, 4 days after i.p. injection of 1 ml sterile 4% brewer modified thioglycollate medium (BD) and a cell count was performed using a hemocytometer. 96 well plates were coated with 400nM of type IV collagen in a total volume of 50ul, incubated for 1 hour at 37°C to allow the collagen to reconstitute, and then left overnight at 4°C. 1X10⁵ cells in 200 ul RPMI were added to the collagen-coated wells and cells were allowed to adhere for 2 hours at 37°C. Non adherent cells were rinsed off with PBS; adherent cells were fixed with 4% paraformaldehyde and stained with 0.5% toluidine blue. The absorbance of the solution in the well was measured in a microtiter plate reader (Molecular Devices, Sunnyvale, California, USA) at 595 nm. This experiment was repeated three times using macrophages isolated from 3 separate mice of each genotype.

**Macrophage invasion assay.**

Peritoneal macrophages were harvested from Ddr1+/+;Ldlr−/− or Ddr1−/−;Ldlr−/− mice by peritoneal lavage with cold RPMI, 3 days after i.p. injection of 1 ml sterile 4% brewer modified thioglycollate medium (BD). Harvested cells were cultured in DMEM containing 10% FCS and 2% penicillin/streptomycin for 24 hours prior to use in a macrophage invasion assay. Briefly, 8 μm transwell filters (Co-star) were coated with 100 ul of a 100 ug/ml collagen IV solution (Sigma), which was allowed to gel for 1 hour at 37°C. Macrophages (10⁵ cells/ well) suspended in 250 ul DMEM containing 2% BSA (serum free medium), were placed in the upper chamber of the transwell which were then placed in 24 well plates containing either serum free medium or 10 ng/ml recombinant mouse MCP-1 (R&D Systems). Invasion was allowed to occur for 4 hours at 37°C after which cells that had migrated through the filter were stained with Coomassie blue and
counted. Five randomly selected 200X fields were counted and averaged for each experiment. The number of cells migrating in response to MCP-1 was normalized to the number migrating toward BSA for each genotype. Data was then expressed as an invasion index relative to Ddr1+/+ macrophages. This experiment was repeated three times using macrophages isolated from 3 separate mice of each genotype.

Statistical analysis.

Data are presented as mean ± SEM. All statistical analysis was carried out using Sigma Stat (SyStat Software Inc.). Pairwise comparisons between transplant groups or genotypes were performed using Student’s t-test. Data that did not fit a normal distribution was analyzed by Mann-Whitney U test for non-parametric comparisons. BrdU pulse data was analyzed using a one way ANOVA with a Tukey post hoc analysis for pairwise comparisons. Statistical significance was determined at p<0.05.

Reference List


Supplemental Table I: Body weight and plasma lipids in DDR1 chimeric mice.

<table>
<thead>
<tr>
<th></th>
<th>$\text{Ddr1}^{+/+} \rightarrow ^{+/+}$</th>
<th>$\text{Ddr1}^{-/-} \rightarrow ^{+/+}$</th>
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<tr>
<td><strong>Body Weight (g)</strong></td>
<td>23.3 ± 2.4</td>
<td>25.6 ± 0.9</td>
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<td><strong>Total Cholesterol (mM)</strong></td>
<td>12.6 ± 0.7</td>
<td>15.0 ± 0.9</td>
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<td><strong>Total Triglycerides (mM)</strong></td>
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**Supplemental Table I:** Body weight and fasting plasma lipids in $\text{Ddr1}^{+/+} \rightarrow ^{-/-}$ and $\text{Ddr1}^{-/-} \rightarrow ^{+/+}$ chimeric mice. Body weight, fasting plasma cholesterol and triglycerides were measured after 12 weeks on the atherogenic diet.

Supplemental Table II: Fasting plasma lipids in $\text{Ddr1}^{+/+};\text{Ldlr}^{-/-}$ and $\text{Ddr1}^{-/-};\text{Ldlr}^{-/-}$ mice.

<table>
<thead>
<tr>
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<th>$\text{Ddr1}^{+/+};\text{Ldlr}^{-/-}$</th>
<th>$\text{Ddr1}^{-/-};\text{Ldlr}^{-/-}$</th>
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</thead>
<tbody>
<tr>
<td><strong>Total Cholesterol (mM)</strong></td>
<td>31.3 ± 3.9</td>
<td>24.6 ± 1.7</td>
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<tr>
<td><strong>Total Triglycerides (mM)</strong></td>
<td>1.8 ± 0.3</td>
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</table>

**Supplemental Table II:** Fasting plasma cholesterol and triglycerides were measured in $\text{Ddr1}^{+/+};\text{Ldlr}^{-/-}$ and $\text{Ddr1}^{-/-};\text{Ldlr}^{-/-}$ mice sacrificed after 2 weeks on the atherogenic diet.
**Supplemental Figure I:** Assessment of male:female chimerism after reciprocal bone marrow transplant. (A) Genomic DNA was isolated from peripheral blood of transplanted chimeric mice and was used to genotype for Ddr1 and Sry, a marker of the Y chromosome. Control lane contains tail DNA from a Ddr1 +/+ male mouse, with the upper band of the doublet representing the Ddr1 knockout allele (ko) and the lower band representing the wild type allele (wt). Genotyping for DDR1 in chimeric mice demonstrated the presence of the donor DDR1 allele in leukocytes, with minimal presence of the host allele. The abundance of Sry was comparable in both groups. (B) Male chimerism was also measured by quantitative PCR for Sry and Gapdh. n=7 animals per group.
Supplemental Figure II: Correlative immunofluorescence to identify the cell type of bone marrow derived cells. Serial cross sections of the aortic sinus were immunostained with antibodies against Mac-2 and SM α-actin and confocal images were correlated with areas of plaque that were analyzed by FISH. Red indicates Y chromosome positive nuclei in the FISH image (A) and the immunofluorescent detection of Mac-2 (B) or SM α-actin (C). Nuclei were stained with Hoescht 33528 and are coloured blue. A grey DIC overlay was added to highlight lesion morphology. From this analysis we conclude that the majority of Y chromosome positive cells are macrophages.