Resident Intimal Dendritic Cells Accumulate Lipid and Contribute to the Initiation of Atherosclerosis

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Rationale: Atherosclerosis is an inflammatory disease in which leukocytes and oxidatively modified lipids accumulate in the arterial intima. Previously, we showed that dendritic cells (DCs) accumulate preferentially in regions predisposed to atherosclerosis in the normal murine aortic intima. The function of these cells in atherogenesis is unknown.

Objective: Our goal was to determine the role of resident intimal DCs in the initiation of atherosclerosis.

Methods and Results: En face immunostaining of nascent atherosclerotic lesions in low-density lipoprotein receptor–deficient (Ldlr<sup>-/-</sup>) mice fed a cholesterol-rich diet for 5 or 10 days demonstrated that foam cells expressed DC markers CD11c, 33D1, and major histocompatibility complex class II. Transmission electron microscopy revealed that the majority of intimal lipid was intracellular. The role of resident intimal DCs in lesion formation was verified by their conditional depletion using transgenic mice expressing the simian diphtheria toxin receptor in CD11c<sup>+</sup> cells. A single injection of diphtheria toxin depleted intimal CD11c<sup>+</sup> DCs by >98% within 24 hours, with 25% and 75% recovery at 1 and 3 weeks, respectively. When bred onto the Ldlr<sup>-/-</sup>background, intimal DC depletion with diphtheria toxin during 5 days of lesion formation reduced the intimal lipid area by 55% relative to undepleted controls. Transmission electron microscopy revealed few foam cells in DC-depleted mice and abundant accumulation of subendothelial extracellular lipid.

Conclusions: Induction of hypercholesterolemia in mice triggers rapid ingestion of lipid by resident intimal DCs, which initiate nascent foam cell lesion formation. (Circ Res. 2010;106:383-390.)

Key Words: atherosclerosis ■ CD11c diphtheria toxin receptor ■ dendritic cells ■ foam cells

In atherogenesis, oxidatively modified lipids, particularly low-density lipoproteins (LDLs), accumulate in the arterial intima, where they are engulfed by myeloid cells. The resulting foam cells constitute a large component of early lesions. Foam cells are derived primarily from monocytes/macrophages, but the majority of research into the nature of foam cells has focused on relatively advanced lesions and has generally used a single monocyte/macrophage marker, such as CD68, MOMA-2, Mac-2, or Mac-3. Some of these markers (eg, CD68) can be expressed by both macrophages and dendritic cells (DCs) and thus cannot differentiate between these cell types. There are, however, several markers expressed preferentially by DCs, including CD11c, major histocompatibility complex (MHC) class II and 33D1 antigen. An in vitro study showed that macrophages, when treated with oxidatively modified lipids, differentiate into foam cells and in the process acquire expression of DC markers. The identity of foam cells is of great significance, because DCs possess distinct functions from macrophages and DC-derived foam cells may retain some or all of their properties.

DCs are found in the intima of human arteries. We and others have shown that DCs reside in the normal murine aortic intima in areas predisposed to atherosclerotic lesion formation and are absent in areas protected from atherosclerosis. We refer to these cells as resident intimal DCs. Macrophages are rare in the normal aortic intima but are abundant throughout the adventitia. DCs are also found in atherosclerotic lesions, in both humans and animal models.

The role of DCs in atherosclerosis is not well understood. DCs isolated from the normal aorta are capable of cross-presenting antigen; however, it is not likely that antigen presentation occurs in the normal intima, because T lymphocytes are very scarce in this location. DCs may migrate to secondary lymphoid organs, where they encounter a wide repertoire of T cells. This phenomenon may decrease during atherogenesis, because hypercholesterolemia impairs migration of DCs to lymph nodes. DCs can also regulate inflammation by producing either proinflammatory or tolerogenic factors in different contexts. Recent studies suggested that lesion...
formation is regulated by myeloid cell homeostasis\(^\text{10,20}\) in the aorta and DCs were shown to regulate plasma cholesterol levels.\(^\text{21}\)

Analysis of ultrastructural features such as tubulovesicular structures suggests that DCs may differentiate into foam cells.\(^\text{22}\) Reduced lesions and intimal DC numbers in hypercholesterolemic as well as normal chemo kinase receptor CX\(_3\)CR\(_1\)\(^{-/-}\) mice suggest that DCs may participate in early stages of atherogenesis.\(^\text{11}\) However, this study did not establish a definitive causal relationship between DCs and lesion development, because CX\(_3\)CR\(_1\) is required for monocyte recruitment\(^\text{23}\) and survival.\(^\text{19}\) We hypothesize that resident intimal DCs may play a key role in the initiation of atherosclerosis by engulfing lipid and differentiating into foam cells.

Conditional depletion of specific cell types can be accomplished using transgenic murine models in which the simian diphtheria toxin receptor CRD feeding, control groups were combined because statistically significant differences between them were not observed.

Mice

All mice were backcrossed onto the C57BL/6 background for a minimum of 6 generations. Wild-type C57BL/6 (DTR\(^+\)), CD11c-DTR (DTR\(^-\)), and Ldlr\(^{-/-}\) mice were from The Jackson Laboratory. DTR\(^+\) mice were maintained in the hemizygous state, and progeny were genotyped as described.\(^\text{24}\) The CD11c-DTR transgene was bred onto the Ldlr\(^{-/-}\) background. DTR\(^+\) and DTR\(^-\) or DTR\(^+\) Ldlr\(^{-/-}\) littermates were used in experiments. Mice were bred and housed under pathogen-free conditions at the University Health Network animal facility. All experiments were performed according to institutional guidelines, as well as Canadian federal and provincial laws for animal protection.

DC Depletion and Induction of Hypercholesterolemia

For systemic depletion of DCs, DTR\(^+\) (age 12 to 16 weeks) or DTR\(^+\) Ldlr\(^{-/-}\) (age 10 to 12 weeks) mice were injected intraperitoneally with DT (4 ng/g body weight, in PBS; List Biologicals). Hypercholesterolemia was induced in 10- to 12-week-old Ldlr\(^{-/-}\) mice by replacing normal chow with CRD (40% fat, 1.25% cholesterol; diet D12108, Research Diets Inc.).\(^\text{26}\) In some experiments, this was immediately after treatment with DT or PBS. Control groups included DTR\(^+\) Ldlr\(^{-/-}\) mice injected with PBS and DTR\(^+\) Ldlr\(^{-/-}\) mice injected with DT. When determining lesion area after 5 days CRD feeding, control groups were combined because statistically significant differences between them were not observed.

En Face Immunostaining and Lipid Staining

En face immunostaining was performed as described (Online Data Supplement). Primary antibodies included anti-CD11c-biotin (1 \(\mu\)g/mL, BD Biosciences), anti-CD45-biotin (0.1 \(\mu\)g/mL, eBioscience), anti-CD68-biotin (0.3 \(\mu\)g/mL, AbD Serotec), 33D1-biotin (20 \(\mu\)g/mL, BD Biosciences), and anti-I-A (MHC II)-PE (2 \(\mu\)g/mL, BD Biosciences). Anti–CD11c-Alexa Fluor 647 (2 \(\mu\)g/mL, BioLegend) was used when costaining with another cell marker. After immunostaining, lipids were stained with Nile red (Sigma) (5 \(\mu\)g/mL. PBS, 30 minutes, 22°C, 3 \(\times\)5-minute PBS washes).

5-Bromodeoxyuridine Labeling

Mice were injected with DT or PBS 72 hours before 5-bromo-2’-deoxyuridine (BrUrd) pulse labeling (Online Data Supplement). Aortic tissue was harvested for analysis after 2 or 24 hours.

Statistical Analyses

The unpaired \(t\) test was used when comparing 2 groups. In experiments with multiple groups, differences were evaluated using 1-way ANOVA, followed by the Tukey–Kramer multiple comparison test. Means±SEM are plotted.

Results

Resident Intimal DCs Accumulate Intracellular Lipid in Nascent Atherosclerotic Lesions

The goal of this study was to determine whether resident intimal DCs found in atherosclerosis-predisposed regions of normal arteries\(^\text{10}\) are capable of engulfing lipid and differentiating into foam cells upon induction of hypercholesterolemia. The expression of DC markers in foam cells of nascent atherosclerotic lesions of Ldlr\(^{-/-}\) mice fed a CRD for 5 or 10 days were assessed (Figure 1). Neutral and polar lipids were stained with Nile red.\(^\text{27}\) At 5 days of CRD, lipid accumulated predominantly extracellular subendothelial lipid accumulation. Taken together, our data demonstrate that in the Ldlr\(^{-/-}\) model resident intimal DCs differentiate into the initial foam cells in nascent atherosclerotic lesions and thus play a key role in atherogenesis.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.
could be visualized in dendrites (Figure 1A and 1B). At 10 days of CRD, the abundance of lipid in CD11c<sup>+</sup> cells increased, consistent with continued lipid uptake (Figure 1E). CD11c<sup>+</sup> foam cells were rounded and lacked dendrites. Transmission electron microscopy (TEM) revealed intracellular vesicles containing stained lipid within foam cells below the endothelium, whereas a relatively small amount of lipid was seen in the extracellular space (Figure 2).

MHC II is a DC marker associated with antigen presentation and is expressed by resident intimal DCs. MHC II was expressed by most CD11c<sup>+</sup> DCs in the normal aortic intima of C57BL/6 mice (Figure 3A), as well as by some CD11c<sup>+</sup> foam cells in Ldlr<sup>−/−</sup> mice fed CRD for 5 days (Figure 3B). At 14 days of CRD, MHC II expression was observed mainly in foam cells at the periphery of the lesion, as well as in DCs outside of the lesion area, whereas in the central lesion, foam cells were mainly MHC II low or negative (Online Figure I).

33D1 is a DC-specific monoclonal antibody, and we recently showed that most resident intimal DCs express this marker. Figure 3C shows that at 5 days of CRD, most CD11c<sup>+</sup> cells coexpress 33D1. Collectively, our data suggest that after initiation of hypercholesterolemia resident intimal DCs engulf lipid and become foam cells expressing DC markers, and the majority of intimal lipid is localized within these cells.

Depletion of Resident Intimal DCs

To confirm that resident intimal DCs differentiate into foam cells, we used CD11c-DTR mice to deplete these cells before initiating hypercholesterolemia. The extent of DC depletion and repopulation has been studied mostly in lymphoid tissues. En face immunostaining of the ascending aortic arch intima for CD11c and CD45 at 24 hours after a single injection of DT revealed that DCs were nearly completely ablated in CD11c-DTR transgene positive (DTR<sup>+</sup>) mice (Figure 4). The ablation of CD45<sup>+</sup> cells confirmed our previous finding that DCs constitute the majority of intimal leukocytes in the normal aorta. Multiple leukocyte types, particularly macrophages, are found in the aortic adventitia, whereas DCs are rare. Consistent with this finding, CD45 staining in the adventitial layer was indistinguishable between DC-depleted and control mice (Figure 4B). Real-time RT-PCR analysis confirmed that DT treatment selectively depletes intimal CD11c<sup>+</sup> DCs at 24 hours but not CD31<sup>+</sup> and intercellular adhesion molecule-2–positive endothelial cells nor CD68<sup>+</sup> adventitial macrophages (Online Figure II).

Silver nitrate staining of endothelial cell junctions revealed that the endothelial cell monolayer remained intact after DC depletion (Online Figure III). The shape and density of endothelial cells was identical in control and DT-treated mice.
Furthermore, the number of intimal nuclei was greater than the number of endothelial cells in the arch LC of control mice (intimal nucleus to endothelial cell ratio was 1.21), whereas following DC depletion the number of nuclei and endothelial cells was equal (ratio was 1.00). We attribute the reduction of intimal nuclei to a loss of resident intimal DCs, but not endothelial nuclei. DT injection did not deplete nuclei in the ascending aortic arch GC, because resident intimal DCs are not located in this region (ratio was 1.00 in both control and DT-treated mice).

DT kills cells by inducing apoptotic cell death, which is characterized by nuclear fragmentation and condensation. At 8 hours following DT injection, these nuclear changes were observed in the intima and were associated with CD11c/H11001 cell membrane blebbing (Online Figure IV). There was no indication of inflammatory response following DT treatment. In fact, real-time RT-PCR revealed that steady-state mRNA levels of proinflammatory genes interleukin (IL)-6 and IL-1/H9252 decreased significantly in the LC of the arch 24 hours following DT treatment, but significant differences in transforming growth factor-β expression levels were not observed (Online Figure V).

**Figure 3.** CD11c^- resident intimal DCs and foam cells express DC markers. Representative en face confocal microscope images of the ascending aortic arch LC demonstrate coexpression of CD11c (red) and MHC II (A and B) or 33D1 (green) (C) by resident intimal DCs in normal C57BL/6 mice (A) and nascent foam cells in Ldlr^-^- mice fed a CRD for 5 days (B and C). Arrows highlight examples of coexpression. Nuclei are blue. Scale bars: 50 μm.

**Figure 4.** Depletion of resident intimal DCs in the mouse aorta. Representative en face confocal microscopy images stained for CD11c (A) or CD45 (B) of the ascending aortic arch harvested from CD11c-DTR mice (C57BL/6 background) 24 hours after injection with either PBS or DT. The LC of PBS-injected mice (A, i; B, i) contained abundant CD11c^- and CD45^- resident intimal DCs with typical dendrites. The LC intima of DC-depleted mice (A, ii; A, iii; and B, ii) contained very few CD11c^- or CD45^- cells, and they were small, round, and lacked dendrites. CD11c^- intimal DCs were not observed in the GC of the aortic arch of either DC-depleted (not shown) or PBS-injected (control) mice (A, iv). CD45^- cells were present in the LC adventitia (Adv) of both PBS-injected (B, iii) and DT-injected (B, iv) mice. Representative images of at least 4 independent experiments are shown. Scale bars: 50 μm.

**Repopulation of Resident Intimal DCs**

After depletion, DCs in lymphoid tissues of DTR^+ mice recover to predepletion levels within several days, whereas in the bladder, recovery is slower. Repopulation of the LC intima in the ascending aortic arch by CD11c^- DCs was determined at different times after DT treatment by en face immunostaining. More than 600 CD11c^- DCs were identified in undepleted controls (PBS-injected DTR^- mice and DT-injected DTR^- mice) (Figure 5A). At 24 hours, <10 DCs were identified, which represents >98% depletion. Gradually, DC numbers increased, with approximately 25% and 75% recovery at 7 and 21 days, respectively.

BrdUrd pulse labeling experiments carried out 3 days after DT treatment revealed that the rate of intimal cell proliferation (analyzed 2 hours after BrdUrd injection) was reduced to levels lower than those found in control mice,
consistent with depletion of intimal DCs (Figure 5B). Monocyte recruitment and proliferation, analyzed 24 hours after BrdUrd injection, did not increase (Figure 5B) and changes in circulating monocyte levels were not found after DT treatment (Online Figure VI). These findings indicate an absence of compensatory intimal cell proliferation or monocyte recruitment induced by DC depletion.

**DC Depletion Reduces Intimal Lipid Accumulation and Foam Cells in Nascent Lesions**

To investigate the role of resident intimal DCs in atherogenesis, the DTR transgene was bred into the Ldlr \(^{-/-}\) background and hypercholesterolemia was initiated by feeding a CRD following DT injection. Control groups included DTR \(^{+}\)Ldlr \(^{-/-}\) mice injected with PBS and DTR \(^{+}\)Ldlr \(^{-/-}\) mice injected with DT. Nascent intimal lesions were analyzed at 5 days by staining for cell markers and lipid. Relative to controls, DT-treated DTR \(^{+}\)Ldlr \(^{-/-}\) mice exhibited a marked reduction in intimal CD11c\(^{+}\) cells and a 55% decrease in aortic surface area stained with Nile red (Figure 6). Quantification of lipid-stained lesion area was performed by 2 different methods and yielded a comparable reduction in DC-depleted mice. The 2 control groups were not significantly different from each other and were thus combined. All groups lost weight within 1 day of DT treatment but regained it subsequently (Online Figure VII, A), and differences between groups were not significant. Biochemical analyses of serum cholesterol and triglycerides revealed comparable levels in experimental and control groups (Online Figure VII, B), consistent with comparable hyperlipidemia.

In control mice without DC depletion, intimal lipid staining in the LC of the ascending arch colocalized very closely with abundant CD11c\(^{+}\) foam cells (Figures 1 and 6). In contrast, the DC-depleted group contained regions in the arch LC that stained for lipid but lacked CD11c\(^{+}\) cells (Figure 6). En face

**Figure 5.** Quantification of resident intimal DC abundance, proliferation, and monocyte recruitment before and following DC depletion. A, Time course shows the abundance of CD11c\(^{+}\) DCs in the LC of the ascending aortic arch harvested from CD11c-DTR transgene \(^{+}\) (DTR\(^{+}\)) mice (C57BL/6 background) before and following DC depletion. Note that the abundance of resident intimal DCs was comparable in DTR\(^{-}\) mice treated with PBS and wild-type (DTR\(^{-}\)) C57BL/6 mice treated with DT. Data are expressed as the total number of CD11c\(^{+}\) cells in the ascending aortic arch and as the percentage relative to control group (DTR\(^{-}\) mice treated with PBS). The means±SEM (n=3 to 4 mice per group) are plotted, and significant differences from the control group are indicated. *P<0.05, **P<0.001. B, Total intimal BrdUrd\(^{+}\) nuclei in the ascending aortic arch were enumerated 2 and 24 hours after BrdUrd pulse labeling in DTR\(^{+}\) mice that were treated with PBS or DT 3 days before BrdUrd injection. The means±SEM (n=4 to 6 mice per group) are plotted, and significant differences are indicated. *P<0.05.

**Figure 6.** Depletion of DCs reduces the surface area of nascent atherosclerotic lesions. A, Composite en face confocal images of the ascending aorta LC harvested from DC-depleted (DT-treated DTR\(^{+}\)Ldlr \(^{-/-}\)) and undepleted (DT-treated DTR\(^{-}\)Ldlr \(^{-/-}\)) mice. DT was injected concurrently with initiation of a CRD that was maintained for 5 days. Accumulated intimal lipid was detected by Nile red staining (red), and CD11c\(^{+}\) cells (green) were detected by immunostaining. Nuclei are blue. Representative images from 7 to 10 experiments are shown. Scale bars represent 500 \(\mu m\). B, Intimal lipid accumulation in undepleted (PBS-treated DTR\(^{+}\)Ldlr \(^{-/-}\) and DT-treated DTR\(^{-}\)Ldlr \(^{-/-}\)) and DC-depleted (DT-treated DTR\(^{+}\)Ldlr \(^{-/-}\)) mice was quantified as described in the Online Data Supplement, and the percentage of the total surface area of the ascending aortic arch stained by Nile red was plotted (means±SEM; n=7 to 10 mice/group). *P<0.05.
immunostaining was performed to investigate whether CD68+ myeloid cells were found in these regions. In control mice, foam cells were costained with CD11c and CD68 (Figure 7A), consistent with conversion of CD11c+ resident intimal DCs into foam cells. At 5 days after DC depletion, a relatively low number of CD11c+CD68+ cells were found in the intima (Figure 7B).

**Intimal Lipid Accumulates Primarily in the Extracellular Space in DC-Depleted Mice**

Because intimal leukocytes were relatively sparse in 5-day lesions of DC-depleted mice, TEM was carried out to determine where intimal lipid accumulates. In contrast to control mice that contained abundant intimal lipid-laden foam cells (Figure 2), foam cells were sparse in the LC intima, and the majority of lipid was located in the extracellular space (Figure 8A and 8B). Abundant particles ranging in diameter from approximately 40 to 70 nm were evident (Online Figure VIII), consistent with aggregated LDL. Occasional foam cells were seen on the luminal surface and based on their location they are likely endothelial cells (Figure 8C).

**Discussion**

DCs have been described in advanced lesions of ApoE−/− mice, but their functions in atherogenesis were not elucidated. The present study reveals that in Ldlr−/− mice, resident intimal DCs readily engulf lipid and rapidly differentiate into foam cells during the initial stages of atherogenesis. In mice in which intimal DCs were depleted, the surface area of lipid accumulation was reduced by 55%, foam cell formation was markedly impaired and lipid accumulated in the extracellular space of the intima.

Previously, monocyte recruitment and differentiation into macrophage foam cells was considered to be one of the earliest steps of atherogenesis. In this study, we demonstrate that engulfment of lipid by resident intimal DCs precedes this step. Atherosclerotic lesions were reproducibly detected by Nile red lipid staining 5 days after initiating hyperlipidemia in Ldlr−/− mice. Virtually all foam cells at this stage expressed the DC marker CD11c and most of them were 33D1+. Some also expressed MHC II, which is consistent with the derivation of these foam cells from resident intimal DCs that express the same DC markers (Figure 3). At 10 to 14 days of CRD, foam cells located in central regions of lesions were enlarged, and CD11c was localized to the periphery of lipid-engorged cells, whereas MHC II expression was diminished (Online Figure I). Early atherosclerotic lesions expand laterally, and DCs at the periphery of lesions differentiated into foam cells and expressed both CD11c and MHC II.

Monocyte recruitment occurs at very low levels at sites predisposed to atherosclerosis in the normal mouse aorta, and resident intimal DCs are likely derived from the recruited monocytes. A recent study in which the bone marrow of Ldlr−/− mice was reconstituted with green fluorescent protein (GFP)-expressing cells independently demonstrated that resident intimal DCs are derived from bone marrow cells. This study also showed that hyperlipidemia induced a progressive
accumulation of bone marrow–derived GFP+ leukocytes in the intima of the ascending aortic arch LC that occupied a maximal surface area by 10 weeks of CRD. However, this study could not distinguish between foam cell formation from resident intimal DCs and newly recruited monocytes because both cell types are derived from the bone marrow and are GFP+. We demonstrated in the present study foam cells expressing DC markers (CD11c, 33D1 and MHC II) at just 5 days of CRD. At this time point, the rate of monocyte recruitment is still relatively low (elevated <2-fold relative to basal recruitment) and does not increase significantly until 2 weeks of CRD feeding.28 These data and our observations that CD11c+ cells at the periphery of lesions take up lipid in dendrites suggest that foam cells are derived primarily from resident intimal DCs. Subsequent experiments with the CD11c-DTR mice established that resident intimal DCs are required for foam cell formation at 5 days of CRD.

The CD11c-DTR mouse model was useful for assessing the functions of resident intimal DCs because a single injection of DT depleted DCs with very high efficiency without an inflammatory response and because repopulation was relatively slow. A report examining the bladder30 suggested that peripheral tissues have significantly slower kinetics of DC repopulation relative to lymphoid organs, where DC repopulation is dependent on robust proliferation of abundant progenitors.33 In contrast, DC repopulation of peripheral tissues may be dependent primarily on monocyte or other DC precursor recruitment rather than proliferation. Our BrdUrd labeling experiments revealed that the low level of intimal cell proliferation decreased following DC depletion and that the recruitment of bone marrow–derived monocytes into the arterial intima continued at a rate equivalent to that in control mice. One day after depletion, the occasional small, rounded CD11c+ cells that were found may be recently recruited monocytes that have induced CD11c expression but have not yet fully differentiated into DCs with dendrites. By day 7 after DT injection, most repopulated intimal CD11c+ cells displayed dendrites (not shown).

Although we did not enumerate the repopulation of intimal DCs following depletion in the setting of hypercholesterolemia, relatively few CD68+CD11c+ cells were detected by en face confocal microscopy at 5 days of CRD feeding. Intimal foam cell formation was markedly diminished in DC-depleted mice. Very few intimal foam cells were found in random sections examined by TEM, which indicates that foam cells were indeed not abundant and suggests that in the absence of DCs, other cell types are not able to take over their lipid uptake function at this early stage of atherogenesis. Collectively, our data establish that resident intimal DCs are required for the formation of initial foam cells during atherogenesis in Ldrr-/- mice.

Confocal microscopy revealed lipid pools in the DC-depleted intima that did not colocalize with CD11c (Figure 6). Examination of sections by TEM revealed that the majority of intimal lipid accumulated in the extracellular space as particles 40 to 70 nm in diameter. These particles were larger than LDL particles, which are 23 nm in diameter,34 suggesting that LDL particles were aggregated, consistent with previous reports.31 Apolipoprotein B in LDL particles can bind to proteoglycans and this property is important for atherogenesis in the setting of hyperlipidemia.35 This phenomenon may also account for the retention of extracellular lipid in the LC intima of DC-depleted mice. The fact that minimal lipid accumulated in the GC intima (Figure 1), which is devoid of resident intimal DCs, suggests that lipid entry into the GC intima is reduced and/or the intrinsic properties of intimal matrix differ between the atherosclerosis-resistant GC intima and the atherosclerosis-susceptible LC intima. Our study suggests that the uptake of lipid by intimal DCs mitigates extracellular lipid accumulation in the LC, because extracellular lipid was much more abundant after DC depletion. However, the aortic surface area occupied by accumulated intimal lipid was reduced by DC depletion.

DCs are known to have both pro- and antiinflammatory roles. For example, DCs promote IL-15 production in response to bacteria-mediated inflammation and lung DCs are critical proinflammatory cells mediating airway inflammation during experimental asthma.36,37 In contrast, depletion of DCs accentuated renal injury in a model of glomerulonephritis possibly because these cells are implicated in the production of IL-10.38 In normcholesterolemic mice, levels of intimal IL-1β mRNA in the LC of the ascending aorta decreased following depletion of DCs, suggesting that resident intimal DCs in the normal aorta may promote a proinflammatory milieu.10 Alternatively, decreased IL-1β mRNA may reflect an antiinflammatory effect induced by apoptotic DCs.39 Inconsistent with this possibility was the fact that mRNA levels of the antiinflammatory cytokines transforming growth factor-β (Online Figure V, B) and IL-10 (not shown) expression did not increase after DT treatment.

A recent study using CD11b-DTR transgenic mice found that repeated depletion of CD11b+ monocytes over a 10-week period reduced atherogenesis in ApoE-/- mice.40 These experiments confirm the importance of monocytes in atherogenesis but were much longer than the 5-day interval that we studied. It is possible that in advanced lesions foam cells form as a result of lipid uptake by newly recruited monocytes that subsequently differentiate into macrophages or DCs. Further investigation will be required to elucidate the functions of DCs versus monocyte/macrophage-derived foam cells during the progression of atherosclerosis. Given the pro- and antiinflammatory functions of DCs, it is conceivable that their role in atherosclerosis is complex.

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Disclosures
None.
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SUPPLEMENTAL MATERIAL

Detailed Methods

*En Face Immunostaining*
Mice were perfused at 100 mm Hg through the left ventricle with ice-cold PBS for 5 min followed by 2% paraformaldehyde in PBS for 10 min. Aortae were harvested and further fixed in 2% paraformaldehyde for 30 min at 4°C. The surrounding adipose tissue was dissected while immersed in cold PBS. Permeabilization was performed using 0.2% Triton X-100 in PBS for 8 min at 22°C. Immunostaining was performed according to the guidelines of the Tyramide amplification kit (PerkinElmer). Essentially, after quenching endogenous peroxidase activity with 3% H$_2$O$_2$, tissue segments were incubated with blocking reagent containing 10 μg/mL nonimmune mouse IgG (Sigma-Aldrich) and IgG corresponding to the species of the primary antibody. Primary antibodies and isotype control IgG were incubated overnight at 4°C. Samples were then incubated with streptavidin-horseradish peroxidase (HRP), followed by FITC-conjugated Tyramide reagent. Lipid was stained with Nile Red$^1$ as described in the Methods Section prior to the nuclear staining with Toto-3 iodide (1 μg/mL PBS; Invitrogen) or Hoechst 33342 (2 μg/mL PBS; Molecular Probes). The arch was opened in a reproducible manner by cutting along the GC as illustrated previously.$^2$ Flattened aortic arch segments were mounted on glass slides using mounting media (Dako Fluorescent; DakoCytomation).

*Confocal Imaging*
Immunoconfocal images were obtained using an Olympus Fluoview 1000 confocal microscope, outfitted with 405, 488, 543, and 633 nm lasers, and Olympus FV10-ASW acquisition software. Bright field imaging was used for photography of silver nitrate-stained samples. Objective lenses include 40x (NA 1.3) and 60x (NA 1.4) oil immersion lenses (Olympus). The time-course of intimal DC recovery was generated by counting all CD11c$^+$ cells in the intima of the ascending aortic arch harvested at the specified time points following DT-injection. Stained cells were viewed and counted using a mercury lamp fitted to the Fluoview confocal microscope. Representative composite immunoconfocal images were created by taking multiple overlapping images with the 40x objective lens and assembling them using Adobe Photoshop.

*Transmission Electron Microscopy*
Mice were perfused through the left ventricle with ice-cold PBS for 2.5 min followed by universal fixative (2.5% glutaraldehyde, 2% PFA in 0.1 M phosphate buffer, pH 7.2) for 10 min at 100 mm Hg. Aortae were harvested and placed in fixative (1% glutaraldehyde, 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2) overnight. The ascending arch was dissected as above, and tissue sections were cut from greater (GC) and lesser (LC) curvatures using a scalpel. Tissues were processed using standard protocols including secondary fixation with osmium tetroxide, cut into 70-80 nm sections, stained with uranyl acetate and Reynolds’ lead citrate, and mounted for transmission electron microscopy (TEM). Electron micrographs were taken using a JOEL JEM-1230 instrument.

*BrdU Pulse Labeling*
BrdU pulse labeling was achieved by intravenous injection of mice with BrdU (2 mg in 0.2 mL sterile PBS, BD Biosciences). For detection of BrdU, immunostaining was performed as described above, with modifications. Following incubation with H$_2$O$_2$, tissue was treated with DNase I for 1.5 h at 37°C, followed by overnight incubation with FITC-conjugated anti-BrdU at 4°C. Tissues were then incubated with an HRP-conjugated anti-FITC antibody (1:300 dilution, Abcam) for 1 hour, followed by incubation with FITC-conjugated Tyramide. Nuclear staining was performed using Hoechst 33342, as above.
Quantification of Lipid Stain
A Zeiss Axiovert 200 Spinning Disk confocal microscope with a Ludl motorized XY stage was used to scan all samples using a 541 nm laser with a 25x (NA 0.8) water immersion objective lens. Composite images were assembled using Volocity acquisition software, and images were analyzed using Adobe Photoshop by manual enumeration of lipid-stained fields and application of the following formula:

\[
\text{% stained area} = \left( \frac{\text{# stained fields}}{\text{total # fields}} \right) \times 100\%
\]

Quantification of each sample was performed in a blinded fashion by two individuals. Lesion quantification was confirmed using a sampling method in which overall lesion size was determined by using Adobe Photoshop to trace around the entire sample area and the general lesion area (simplified to an oval shape), quantify pixel number in each case, and express the result as a percentage. The percent stained area within the lesion was then determined by taking sample images using an Olympus Fluoview 1000 confocal microscope with a 40x objective lens (as described above), in 5-7 regions of the lesion, selected consistently among samples. Percent stained area within these images was quantified using Image J software (NIH), and averaged for each sample. The total percent stained area was then calculated as shown below:

\[
\text{Total % stained area} = \left( \frac{\text{% overall lesion size}}{\text{% stained area within lesion}} \right) / 100\%
\]

Graphs were generated using the sampling method of quantification.

Harvesting of Mouse Aortic Cells for Real-time RT-PCR
Cells were harvested from mouse aortic intima (LC, GC, and descending thoracic regions) and adventitia for mRNA expression analysis. This was achieved by perfusing mice through the left ventricle with 20 mL of ice-cold PBS containing 1% heparin, using a sterile syringe and a 23-gauge needle. The ascending aortic arch and descending thoracic aorta were harvested, and adipose tissue was removed while immersed in ice-cold PBS + 1 mM aurintricarboxylic acid (Sigma). Random samples of adventitia were removed from the aortic arch and stored in ice-cold PBS + 1mM aurintricarboxylic acid. Segments of aorta were opened and pinned with the endothelial surface upwards on a dish with a black silicone base. Nuclei were stained for 5 min at 22°C with modified Harris hematoxylin solution (Accustain; Sigma-Aldrich), rinsed, and digested for 8 min at 37°C with Liberase Blendzyme 2 (1:100; Roche Diagnostics) in Ca²⁺/Mg²⁺-containing PBS. Intimal cells from the LC, GC, and from the descending thoracic aorta (excluding the ostia) were gently scraped with a 25-gauge needle and transferred with a pipetman into sterile microcentrifuge tubes. For each experiment, intimal or adventitial cells were pooled from 3 aortae. We estimate that <1000 intimal cells were harvested per pooled sample.

RNA Isolation and Reverse Transcription
Total RNA was isolated from harvested cells using the PicoPure RNA Isolation Kit (Arcturus Inc.) according to kit protocol. Reverse transcription reactions were performed using random primers in conjunction with the SuperScript III RT Kit (Invitrogen).

Real-time PCR
Primer pairs (supplemental Table) were designed using Primer Express software (Applied Biosystems) and purchased from Operon Biotechnologies, Inc. (Huntsville, AL). cDNA was diluted 1/20 in ddH₂O, and real-time PCR was performed on a LightCycler 480 (Roche) using Roche SYBR Green I Mastermix. The cycle settings used were: 95°C for 5 min followed by 45 cycles of 95°C, 60°C, and 72°C for 10, 20, and 30 seconds, respectively. Standard curves were generated for each primer set by real-time PCR of 1/10 serial dilutions of reference cDNA (generated from mouse heart, liver and lung total RNA). All gene expression data were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) and acidic
ribosomal phosphoprotein (ARP). For each gene, normalized values were compared to the corresponding value for the PBS-injected DTR⁺ mouse LC region, which was assigned a value of 1.

Silver Nitrate Staining of Endothelial Cell Junctions
Mice were perfused through the left ventricle as follows: PBS (5 min), AgNO₃ (0.125% in H₂O, 30 sec), PBS (1 min), and finally 2% PFA (in PBS, 10 min). Aortae were harvested and fixed for an additional 10 min in ice-cold 2% PFA, and ascending arches were dissected as described.¹ Nuclei were counterstained with propidium iodide (4 μg/mL in PBS; Sigma).

Serum Biochemical Analysis
Blood was collected at time of sacrifice by cardiac puncture, then allowed to clot at 22°C for 30 min. Serum was obtained by microcentrifuging samples as 12,000 rpm for 10 min at 4°C, then collecting the supernatant. Serum samples were stored at -80°C, and analysis of cholesterol and triglyceride concentrations was performed by Vita-tech Laboratories (Markham, ON).

Flow Cytometry
Blood was collected by cardiac puncture at time of sacrifice of DTR⁺ mice or C57BL/6 mice 24 h after DT treatment. Blood leukocytes were counted and stained with primary antibodies, anti-Ly6C-biotin (Biomedicals, AG) and anti-CD115-PE (eBioscience), followed by streptavidin-APC (BD Biosciences). Data were acquired using a flow cytometer (Cytomics FC500 MPL, Beckman Coulter) and analysis was performed using Flowjo software version 7.5 (Tree Star Inc.). The abundance of individual monocyte subtypes in the circulation was calculated based on percentages determined by flow cytometry multiplied by the total number of blood leukocytes determined with a hemocytometer.
Supplemental References


<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
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**Supplemental Table I.** Real time RT-PCR primers. All primer pairs span intron/exon boundaries or are located in adjacent exons.
Supplemental Figure I. CD11c⁺ intimal cells (red) co-express MHC II (green) in 14-day lesions. Representative en face confocal image of the ascending arch LC from a Ldlr⁻/⁻ mouse fed a CRD for 14 days shows CD11c⁺MHC II⁺ foam cells (arrows) at the periphery of a lesion, and CD11c⁺MHC II⁺ DC (arrowheads) adjacent to the lesion. The central area of the lesion contains CD11c⁻MHC II low or negative foam cells (asterisks). Scale bars represent 50 μm.
Supplemental Figure II. Real time RT-PCR analysis of cell marker mRNA expression in the aortic intima and adventitia 24 h after treatment of DTR+ mice with DT or PBS. (A) Expression levels of CD31 and intercellular adhesion molecule-2 (ICAM-2), which are relatively specific for endothelial cells, were comparable between the LC and GC intima and between DC-depleted and undepleted mice. (B) The myeloid cell marker CD68 was expressed predominantly in the LC intima and adventitia (Adv), and at low levels in the GC intima. DT treatment significantly lowered CD68 levels only in the LC intima. Expression of CD11c was relatively high in the LC intima and was decreased by DT treatment. In contrast, CD11c expression in the GC intima and adventitia was low and was not altered by DT treatment. The means ± SEM (n = 3 mice per group) are plotted and significant differences are indicated (*P < 0.05).
Intimal nuclear density in the mouse aorta

<table>
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<tr>
<th>Genotype/treatment</th>
<th>Aortic region</th>
<th>Intimal nuclei per mm²</th>
<th>EC per mm²</th>
<th>Nuclei to EC ratio</th>
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<td>DTR⁻ /DT</td>
<td>LC</td>
<td>2336 ± 231</td>
<td>1932 ± 196</td>
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<td>DTR⁻ /DT</td>
<td>GC</td>
<td>1837 ± 162</td>
<td>1833 ± 159</td>
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<td>2083 ± 83</td>
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<td>DTR⁺/DT</td>
<td>GC</td>
<td>1942 ± 110</td>
<td>1939 ± 111</td>
<td>1.00</td>
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Supplemental Figure III. The endothelial cell monolayer remains intact after depletion of DC and the ratio of intimal nuclei to endothelial cells decreases in the LC region. DIC images show endothelial cell (EC) junctions stained with silver nitrate and nuclei stained with propidium iodide in the LC and GC intima of DTR⁻ and DTR⁺ C57BL/6 mice 24 h after treatment with DT. Nuclei in the LC intima of DTR⁻ mice were more abundant, occasionally displayed kidney-shaped morphology typical of myeloid cells and overlapped endothelial junctions (arrows). After treatment of DTR⁺ mice with DT, EC in the LC region maintained their cobblestone shape, but the abundance of intimal nuclei was reduced. Scale bars represent 50 μm. The table shows the density of intimal nuclei and EC, and the ratio of intimal nuclei to EC. Data are presented as means ± SEM (n = 4 mice per group).
Supplemental Figure IV. DT triggers apoptosis of intimal CD11c⁺ cells in DTR⁺ mice. Eight hours after DT treatment of DTR⁺ mice, cell membrane blebbing (arrowhead) and nuclear condensation (arrow) were observed in CD11c⁺ cells (green) located in the LC intima of the ascending aortic arch. A representative image is shown. The scale bar represents 50 μm.
Supplemental Figure V. Real time RT-PCR quantification of pro- and anti-inflammatory cytokine mRNA expression in the aortic intima and adventitia. DTR⁺ mice were treated with DT or PBS for 24 h prior to harvesting of RNA. (A) Pro-inflammatory gene expression correlated with the abundance of intimal DC. mRNA expression of interleukin (IL)-6, tumor necrosis factor (TNF)-α and IL-1β decreased in the LC intima following DC depletion by DT. TNF-α and IL-1β both demonstrated low to undetectable expression in the GC intima and the adventitia (Adv), while IL-6 displayed relatively high, but variable expression in all regions. (B) Transforming growth factor (TGF)-β mRNA expression was comparable in all regions, and did not correlate with the abundance of intimal DC. A trend towards increased expression of TGF-β was detected in the LC intima following DT treatment, although it did not reach statistical significance. No difference in TGF-β expression was observed in the GC intima or the adventitia. Means ± SEM (n = 3 mice per group) are plotted and significant differences are indicated (* P < 0.01, ** P < 0.001).
Supplemental Figure VI. Circulating mouse monocyte numbers do not change after DT-treatment. DTR⁻ and DTR⁺ C57BL/6 mice were injected with DT and blood was collected after 24 h and analyzed as described in Supplemental Methods. (A) Representative flow cytometry plots illustrate gating of blood leukocytes (WBC) harvested after DT injection. The percentages of Ly-6C<sup>high</sup>, Ly-6C<sup>low</sup> and Ly-6C<sup>neg</sup> CD115<sup>+</sup> monocytes are indicated. (B) The absolute numbers of circulating monocyte subsets in DTR⁻ and DTR⁺ mice treated with DT are plotted. Four independent experiments were performed, and data were derived by multiplying the total leukocyte count by the percentage of each monocyte subtype, determined by flow cytometry, as is illustrated in panel A. The means ± SEM are presented.
Supplemental Figure VII. All groups of hypercholesterolemic mice exhibited equivalent body weight and serum biochemistry. (A) The body weight over the course of the experiment (DT or PBS treatment and CRD) is shown. (B) Serum triglycerides and cholesterol levels obtained on day 5 are plotted. Data represented as means ± SEM (n = 4-7 mice per group). There were no significant differences between the groups.
Supplemental Figure VIII. Determination of aggregated lipid particle diameters. The same transmission electron micrograph as in Figure 8B is shown with the diameters of random particles indicated. The image shows the intima in the LC of the ascending aorta that was harvested from a DT-treated DTR\textsuperscript{+} Ldlr\textsuperscript{−/−} mouse after 5 days of feeding a CRD. Aggregated lipid particles are trapped in the extracellular matrix below the endothelial cell monolayer (EC) and above the internal elastic lamina (IEL). The scale bar represents 500 nm.