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Flt3 Signaling-Dependent Dendritic Cells Protect against Atherosclerosis

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SUMMARY

Early events in atherosclerosis occur in the aortic intima and involve monocytes that become macrophages. We looked for these cells in the steady state adult mouse aorta, and surprisingly, we found a dominance of dendritic cells (DCs) in the intima. In contrast to aortic adventitial macrophages, CD11c$^+$MHC II^{hi} DCs were poorly phagocytic but were immune stimulatory. DCs were of two types primarily: classical Flt3-Flt3L signaling-dependent, CD103$^+$CD11b$^-$ DCs and macrophage-colony stimulating factor (M-CSF)-dependent, CD14$^+$CD11b$^+$DC-SIGN$^+$ monocyte-derived DCs. Both types expanded during atherosclerosis. By crossing *Flt3*$^{-/-}$ to *Ldlr*$^{-/-}$ atherosclerosis-prone mice, we developed a selective and marked deficiency of classical CD103$^+$ aortic DCs, and they were associated with exacerbated atherosclerosis without alterations in blood lipids. Concomitantly, the *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice had fewer Foxp3$^+$ Treg cells and increased inflammatory cytokine mRNAs in the aorta. Therefore, functional DCs are dominant in normal aortic intima and, in contrast to macrophages, CD103$^+$ classical DCs are associated with atherosclerosis protection.

INTRODUCTION

Large numbers of inflammatory and immune cells are found in the aorta during several vascular diseases including atherosclerosis, giant cell arteritis, and aortic aneurysm (Hansson and Libby, 2006; Kuivaniemi et al., 2008; Weber et al., 2008; Weyand et al., 2004). In addition to atherosclerosis being a chronic inflammatory disease of the aorta and large blood vessels (Ross, 1999), it is also at least in part an immune disease (Hansson and Jonasson, 2009; Ross, 1999). The precise definition of the development and function of immune cells in normal and diseased vessels is therefore increasingly important.

In the initial stages of atherosclerosis, blood monocytes enter the subintimal space and take up lipids to become foam cells (Lessner et al., 2002). T cells also accumulate in the atherosclerotic plaque (Gown et al., 1986; Hansson et al., 1989; Jeon et al., 2010; Jonasson et al., 1986). Previous studies indicate that a major autoantigen for human T cells from fresh intimal plaques is oxidized LDL (oxLDL), which upon activation release atherogenic chemokines, like monocyte chemoattractant protein-1 (MCP-1) (Hansson et al., 2002; Stemme et al., 1995). Alternatively regulatory T (Treg) cells are present and are reported to attenuate atherosclerosis (Ait-Oufella et al., 2006; Mallat et al., 2003; Mor et al., 2007; Sasaki et al., 2009; Takeda et al., 2010). However, a bidirectional link between monocytes and T cells is perplexing because monocytes are poor immune-stimulating cells (Van Voorhis et al., 1983) until they differentiate into dendritic cells (DCs) (Romani et al., 1994; Sallusto and Lanzavecchia, 1994). Interestingly, transfer of DCs loaded with oxidized LDL can protect rather than enhance atherosclerosis (Habets et al., 2010). Therefore, if lipoproteins mediate an interaction between antigen-presenting cells and T cells in the atherosclerotic intima (Sun et al., 2010) (Benagiano et al., 2005), it becomes important to know the extent and type of antigen-presenting cells in this site in vivo and to find out whether some have the capacity to protect against disease.

The expression of surface molecules MHC class II (MHC II), CD11b, CD11c, and CD68 has been used for identifying presumptive macrophages and DCs in mouse aorta (Galkina et al., 2006), whereas staining for CD68 and CD11c has localized these cells to the intima of the lesser curvature of the aortic arch and openings of arterial branches, which are atherosclerosis-prone areas (Jongstra-Bilen et al., 2006). Expression of CD11c also has been used to mark presumptive DCs that accumulate lipid during atherosclerosis (Cybulsky and Jongstra-Bilen, 2010; Paulson et al., 2010). It is proposed that aortic DCs are inflammatory, derived from monocytes, and increased during atherosclerosis (Liu et al., 2008).

However, the early research discriminating DCs from monocytes did not employ the functional and developmental criteria that are valuable for identifying and understanding cell function (Cheong et al., 2010a; Liu et al., 2009), and these criteria might likewise be critical to understand atherosclerosis and develop new approaches and tools for the field.

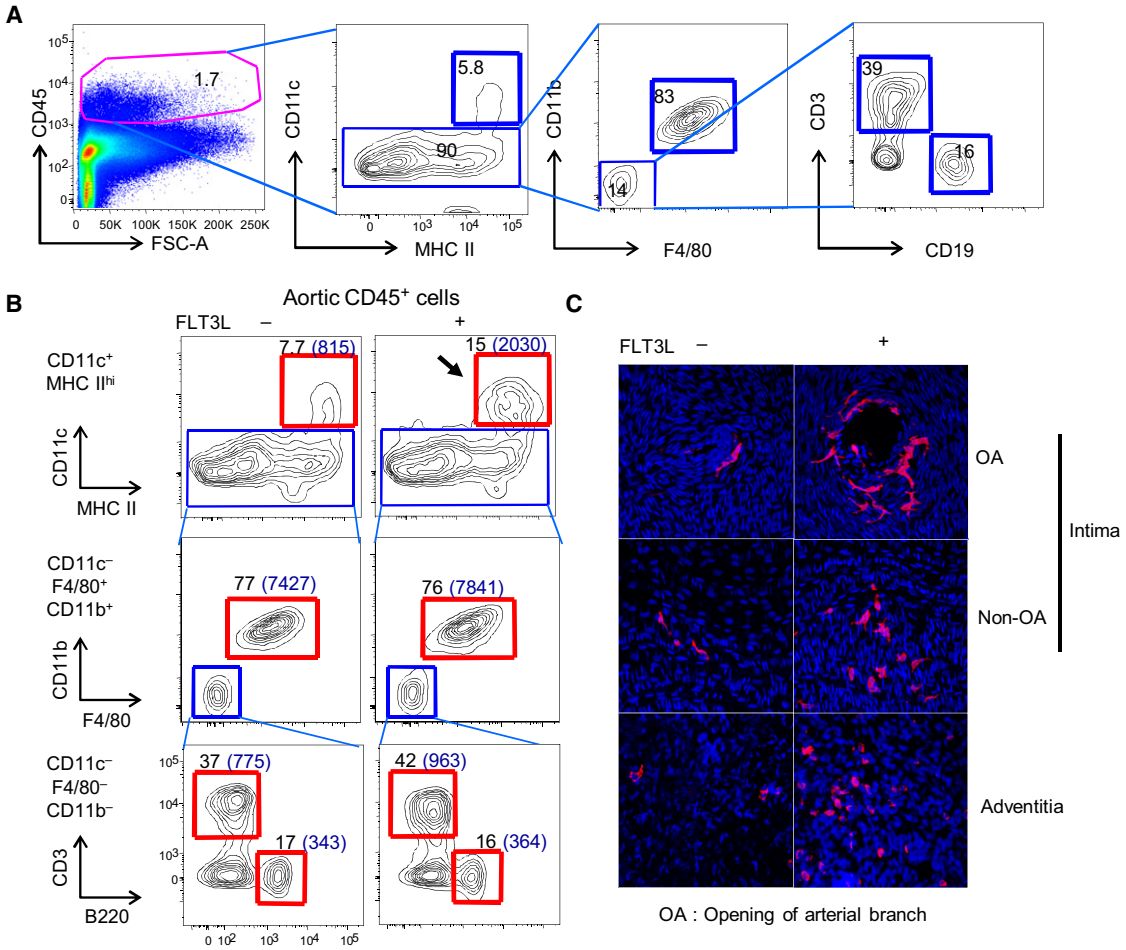

Figure 1. Presence of Flt3L-Dependent DCs in Aortic Intima

(A) FACS procedures for analysis of aortic leukocytes in enzyme digested aorta. The liberated cells were stained for CD45, CD11c, CD11b, F4/80, MHC II, CD3, and CD19 and analyzed with a LSRII instrument (BD).

(B) Ten micrograms of Flt3L was injected i.p. for nine consecutive days, and then aortic leukocytes were analyzed by FACS to show selective increase in $CD11c^+MHC\ II^{hi}$ cells (arrow, top right).

(C) As in (B), showing marked increase in intimal $CD11c^+$ cells (red) by en face immunostaining of aorta from Flt3L treated mouse. The figures in each section are representative of at least three experiments.

Here, we localize and characterize aortic mononuclear cells, emphasizing newly appreciated evidence for two major pathways of DC development, one driven by the cytokine fms-like thyrosine kinase 3 ligand (Flt3L) and its receptor (Flt3) acting on monocyte-independent precursors (Liu et al., 2009; Naik et al., 2006; Onai et al., 2006) and another from monocytes independently of Flt3L-Flt3 (Cheong et al., 2010a). Surprisingly we find that the nonlymphocytes of the subintima in the steady state are mainly comprised of poorly phagocytic, immune stimulatory DCs, not monocytes, and that these are derived from both major developmental pathways: Flt3-Flt3L dependent classical DCs, and M-CSF dependent monocyte-derived DCs. Both cell types are found in diseased mouse aorta as well. Whereas most types of immune cells are thought to exacerbate atherosclerosis (Stoneman et al., 2007; Sun et al., 2007b; Whitman et al., 2004; Zhou et al., 2000), we find that Flt3-deficient low-density lipoprotein receptor knockout ($Ldlr^{-/-}$) mice have lower numbers of classical DCs and develop more severe atherosclerosis relative to control $Ldlr^{-/-}$ mice. Flt3 deficient $Ldlr^{-/-}$ mice have less Tregs and more inflammatory cytokines in the aorta. Our findings indicate that classical DCs, in contrast to monocytes and macrophages, have an atheroprotective function.

RESULTS

Flt3L treatment Selectively Increases $CD11c^+MHC\ II^{hi}$ Cells in the Aorta

To better identify different cell types in the normal mouse aorta, we developed a multicolor flow cytometry procedure for cell suspensions from the aortic root (valves and aortic sinus) and both ascending and descending aorta. The aorta was dissociated with an enzyme mixture and then stained with monoclonal antibodies for dissecting the subset of leukocytes. $CD45^+$ leukocytes (Figure 1A, top left) were further defined with CD11c and

MHC II in looking for CD11c and MHC II-high, presumptive DCs (Figure 1A, second from left). Among the CD45$^+$ leukocytes (average yield about 10,000/aorta; ~1%–2% of the aortic cell suspension), we observed a minor fraction of CD11c$^+$MHC II^{hi} cells (Figure 1A and Figure S1A available online), which we showed previously had immune stimulating and cross-presenting capacities similar to DCs from lymphoid tissues (Choi et al., 2009). The CD11c$^-$ cells were comprised of a major population that double labeled for F4/80 and CD11b (Figure S1A). Many of the latter cells expressed lower surface MHC II molecules compared with CD11c$^+$MHC II^{hi} cells (MFI: 1.3 × 10^4 versus 5.2 × 10^4, Figure S1B). MHC II expression on these F4/80$^+$ CD11b$^+$CD11c$^-$ cells was comparable to B cells (Figure 1A and Figure S1A). After immunolabeling, in contrast to the CD11c$^+$ cells in the intima of normal aorta (Choi et al., 2009), CD11c$^-$ but F4/80$^+$ cells were abundant in adventitia (Figure S1C).

To gain developmental evidence that the CD11c$^+$MHC II^{hi} cells were DCs, we treated mice with 10 μg of Flt3L for nine consecutive days as described in previous studies showing the expansion of DCs but not macrophages in response to Flt3L in lymphoid and nonlymphoid tissues (Ginhoux et al., 2009; Waskow et al., 2008). Aortic CD11c$^+$MHC II^{hi} cells were increased 2.5- to 3-fold in Flt3L-treated mice (Figure 1B, arrow), whereas other populations were slightly increased or not changed (Figure 1B). When we checked for the distribution of the Flt3L-mobilized DCs by en face immunostaining, the CD11c$^+$ cells were markedly increased in both the intima and adventitia of Flt3L-treated mice (Figure 1C). This approach provides developmental evidence that CD11c and MHC II-high cells in the aorta include DCs whereas the CD11c$^-$ cells are primarily macrophages expressing higher amounts of F4/80 and CD11b and lower expression of MHC II than the DCs (Figures S1A and S1B).

Aortic Dendritic Cell and Macrophage Populations Can Be Distinguished by Functional Criteria

To test whether F4/80$^+$CD11b$^+$ cells had the phagocytic activity of macrophages, we bathed aortic cell suspensions in serum-containing RPMI medium with 0.5 μm fluorescent YG microspheres, and 70 min later, we analyzed the phagocytic activity with ImageStream analysis and flow cytometry; the former allows one to visualize single cells for uptake and other cell surface markers simultaneously. CD11c$^+$MHC II^{hi} DCs took up 0–1 bead (Figure 2A, top three rows), whereas phagocytic activity was clearly evident in the CD11c$^-$CD11b$^+$MHC II$^-$ macrophages (Figure 2A, middle three rows). The CD45$^+$ CD11c$^-$CD11b$^-$ fraction was also nonphagocytic (Figure 2A, lower three rows). These findings were confirmed by FACS analysis (Figure 2B).

To identify cell populations with the immune-stimulatory capacity of DCs, we sorted cell fractions and tested them for stimulation of T cells in the primary mixed leukocyte reaction (MLR) (Figure 2C). Only the CD11c$^+$MHC II^{hi} aortic cells were strong stimulators of proliferation in both CD4$^+$ and CD8$^+$ T cells (Figure S2) and similar to that observed with splenic DCs (Figure 2C). In contrast the macrophage and lymphocyte fractions, which included MHC II$^+$ macrophages and B cells, were nonstimulatory (Figures 2C and S2). Therefore, by functional criteria, aortic cell suspensions have a major population of phagocytic, nonimmune stimulatory macrophages and a minor population of poorly phagocytic, immune stimulatory DCs (Steinman and Cohn, 1974; Steinman and Witmer, 1978).

CD11c$^-$F4/80$^+$CD11b$^+$ Aortic Cells Are Selectively Ablated by Two Monocyte Depletion Methods

To evaluate the effects of monocyte depletion, we first injected diphtheria toxin (DT) into CD11b-DTR mice, which specifically depletes monocytes (Duffield et al., 2005; Stoneman et al., 2007). When we checked aortic CD45$^+$ leukocytes, the CD11c$^-$ phagocytes with high expressions of F4/80 and CD11b were markedly decreased, whereas CD11c$^+$MHC II^{hi} DCs were still intact (Figure 3A, arrows). The resistance of the DCs to DT was to some extent surprising because, as will be shown below, some aortic DCs do express intermediate levels of CD11b, as is also the case with CD8$^-$CD11b$^+$ spleen DCs. However, we also observed that splenic DCs but not splenic monocytes and macrophages were resistant to DT treatment (Figure S3A). Therefore, this depletion method depletes monocytes and macrophages, but does not deplete the DC subset in spleen or aorta that expresses CD11b. This surprising resistance of DCs in spleen and aorta of CD11b-DTR mice to DT treatment may reflect lower and/or less stable DTR expression, and interestingly CD11b$^+$ neutrophils also have been reported to be resistant to DT depletion in these transgenic mice (Duffield et al., 2005).

To analyze development at the level of hematopoietins, we also investigated *op/op* mice that lack the M-CSF hematopoietin for monocytes and macrophages but not most DCs in lymphoid tissues in steady state (Witmer-Pack et al., 1993). CD11c$^-$F4/80$^+$CD11b$^+$ macrophages in the aorta of *op/op* mice were decreased by ~90% compared with WT control mice (Figure 3B, arrow). However, the other populations remained intact (Figure 3B and Figure S3B). Thus, the results from CD11b-DTR and *op/op* mice suggest a monocyte origin of CD11c$^-$F4/80$^+$CD11b$^+$ cells.

Two Major Subsets of Dendritic Cells in Normal Mouse Aorta

To extend the above genetic approaches to DCs, we first determined whether aortic DCs were comprised of subsets, as is the case in many lymphoid and nonlymphoid tissues (reviewed in Merad and Manz, 2009). When we stained the intimal CD11c$^+$ cells in intact aortic tissue for CD11b, there appeared to be two subsets, i.e., CD11b-negative and -positive, CD11c$^+$ cells (Figure 4A). By FACS analysis, we could also see CD11b$^+$F4/80$^+$ and CD11b$^-$F4/80$^-$ DC subsets, as well as a small CD11b$^+$F4/80$^-$ population. The low frequency of the latter cells prevented further study at this time (Figure 4B). B220$^+$CD19$^-$ plasmacytoid DCs also were scarce (data not shown).

To verify that the CD11b$^-$F4/80$^-$ aortic DCs were comparable to what are termed CD8α$^+$ DCs in lymphoid organs, we examined these DCs for their marker expression. We found that aortic DCs did not express CD8, CD205, and 33D1 (data not shown). However, when we tested for expression of CD103, a ligand for E-cadherin expressed by most epithelial cells and also a marker for CD11b$^-$ DCs in many tissues (Annacker et al., 2005; Edelson et al., 2010; Johansson-Lindbom et al., 2005; Merad and Manz, 2009; Sung et al., 2006), most CD11b$^-$F4/80$^-$ DCs expressed

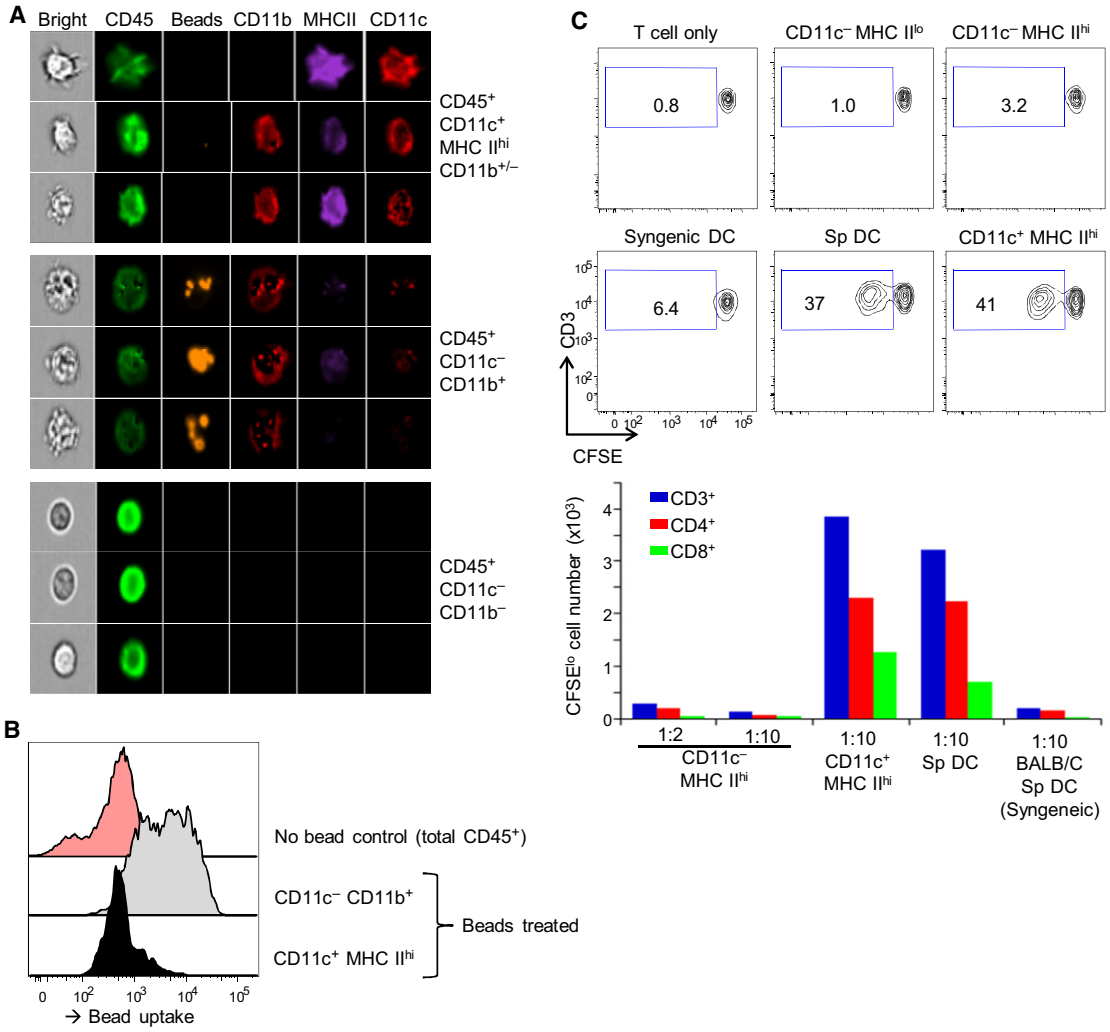

Figure 2. Distinguishing Aortic Dendritic Cell and Macrophage Populations by Functional Criteria

(A and B) Uptake of 0.5 μm microspheres (YG, Molecular Probes) after 70 min exposure of aortic segments that had been digested with the enzyme mixture by (A) ImageStream and (B) FACS.

(C) Stimulation of $CD3^+$ CFSE-labeled T cells in the mixed leukocyte reaction (methods) by different populations of aortic cells DCs, e.g., $CD45^+CD11c^+MHC\ II^{hi}$ DCs (lower right) and macrophages, ($CD45^+CD11c^-MHC\ II^+$, $CD45^+CD11c^-MHC\ II^-$) upper row. Splenic (Sp) $CD11c^+MHC\ II^{hi}$ DCs were isolated from C57BL/6 and BALB/C (syngeneic) mice for positive and negative controls of the MLR, respectively. CFSE dilution was analyzed 5 days later. Each figure is representative of three experiments.

CD103 in normal aorta (Figure 4C, blue), and this molecule was not found on any other population of aortic leukocyte (data not shown). Furthermore, these $CD103^+$ DCs selectively expressed Langerin (CD207) (Kissenpfennig et al., 2005; Cheong et al., 2007) (Figure S4).

When we separately examined aortic cells from the aortic sinus and the rest of the aorta (aortic arch and thoracic aorta), most $CD103^+CD11b^-F4/80^-$ DCs were from the aortic sinus, but these cells were also present in other regions of the aorta in smaller amounts (Figure 4D, blue population). To further define the exact location of these cells in the aortic wall, we analyzed the aortic DC populations after removing the adventitial tissue. Surgical removal of adventitial tissues increased the frequency of $CD11c^+$ DCs in the recovered $CD45^+$ leukocytes, but decreased the frequency of macrophages. These results indicate that DCs are more localized to the intima than adventitia whereas macrophages are mainly localized in the adventitia (Figure 4E). We tentatively concluded that the aortic intima had two major subsets of DCs (Figure S4B): $CD103^+CD11b^-F4/80^-$ and $CD103^-CD11b^+F4/80^+$ populations.

$CD11b^-$ $F4/80^-$ Aortic DCs Selectively Express Flt3 and Respond to Flt3 Ligand

To extend prior work showing expression of Flt3/CD135, the receptor for Flt3L on classical DCs, we found unexpectedly that CD135 was detected primarily on $CD11b^-$ aortic DCs by FACS, and not all DCs (Figure S5A, population I), as anticipated from prior work (Liu et al., 2009; Naik et al., 2006; Onai et al., 2006). To pursue this finding, we investigated Flt3-deficient ($Flt3^{-/-}$) mice and found that the total $CD11c^+MHC\ II^{hi}$ DCs

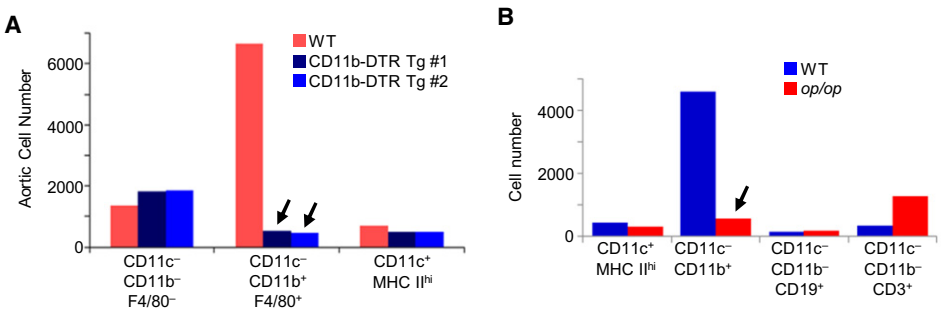

Figure 3. Ablation of CD11c⁻ F4/80⁺ CD11b⁺ Aortic Cells by Two Monocyte Depletion Methods

(A) Selective depletion of aortic CD11c⁻CD11b⁺F4/80⁺ cells by 0.5 μg DT i.v. (25 ng/g weight) after 24 hr in CD11b-DTR transgenic mice.

(B) Marked depletion of CD11c⁻CD11b⁺F4/80⁺ cells (arrow) in M-CSF deleted *op/op* mice. Each figure is representative of at least three experiments.

were slightly decreased but the CD11b⁻F4/80⁻ population was selectively and markedly decreased (Figure 5A, arrow, middle row). Likewise, CD103⁺ DCs were also markedly reduced in *Flt3*$^{-/-}$mice (Figure 5A, arrow, lower row). When we tested responses to Flt3L treatment, F4/80⁻ DCs, both CD11b⁻ and CD11b⁺ DC subsets, were markedly increased (Figure 5B, upper panels), whereas CD11c⁻F4/80⁺CD11b⁺ macrophages were not (Figure 5B, lower panels). The Flt3 requirement was cell intrinsic because when we injected 1:1 mixtures of C57BL/6 (B6) WT (CD45.2) and *Flt3*$^{-/-}$ (CD45.1) marrow into lethally irradiated CD45.2 B6 (5.5 Gy × 2, 3 hr apart) mice, ~25% of blood leukocytes were from the *Flt3*$^{-/-}$ donor at 6 weeks. When we checked the aortic CD11c⁺MHC II^{hi} populations, we found that marrow cells from *Flt3*$^{-/-}$ mice failed to make a CD11b⁻F4/80⁻ population, indicating that the generation of CD11b⁻F4/80⁻ cells is highly dependent on Flt3 signaling (Figure S5B). Corroborating our findings is the fact that Flt3L-deficient mice also selectively lack CD103⁺DCs (Figure S5C). These data indicate that CD11b⁻F4/80⁻CD103⁺ cells in the aorta are typical Flt3-dependent DCs, but the CD11b⁺F4/80⁺ DCs are not.

CD11b⁺ F4/80⁺ CD103⁻ DCs Express CD14 and DC-SIGN and Are M-CSF Dependent

To determine whether CD11b⁺F4/80⁺CD103⁻ DCs were related to monocyte-derived DCs, as described in a recent paper (Cheong et al., 2010a), we added CD14 as a marker. We found the CD11b⁺CD103⁻ cells expressed the CD14 coreceptor for TLR4 and also that injected DC-SIGN antibody (BMD30) (Cheong et al., 2010b) could label these cells (Figures 6A and 6B). Furthermore, in *op/op* mice, this population was dramatically decreased, indicating that these cells also require M-CSF (Figures 6C and S6A). These results indicate that the CD11b⁺ CD103⁻ population are not the equivalent of CD8α⁻ DCs, which are monocyte independent, but are consistent with an origin from monocytes.

Previous studies (Liu et al., 2009; Naik et al., 2006; Onai et al., 2006) showed that classical DCs originate from DC precursors with low expression of CX_3CR1, whereas monocytes and monocyte-derived cells have higher CX_3CR1 expression. We next checked for CX_3CR1 expression of each aortic DC population. CX_3CR1 was expressed only by CD11b⁺F4/80⁺ DCs and CD11b⁺F4/80⁻ DCs and absent from CD11b⁻F4/80⁻ population (Figures S6B and S4B).

To confirm that these CD14⁺CD11b⁺ cells were monocyte-derived DCs, functional tests were done. Both CD14⁺ CD103⁻ and CD14⁻ CD103⁺ DCs were poorly phagocytic (Figure S6C, also see Figure 2A for morphology). When we tested their capacity to stimulate T cells in the MLR, the CD14⁺ and CD14⁻ cells were as immune stimulatory as splenic DCs (Figure 6D). Thus, CD11c⁺MHC II^{hi} DCs of the aorta are of two types, Flt3 and M-CSF dependent, and both are poorly phagocytic and strongly stimulatory for T cell responses.

Both DC Subsets Are Increased in Atherosclerotic Aorta and Flt3-Dependent DCs Are Atheroprotective

To examine aortic DC subsets in the atherosclerotic aorta, we used *Ldlr*$^{-/-}$ mice that were fed a high cholesterol diet for 16 weeks. Initially, we double-stained whole mount of the atherosclerotic aorta with antibodies to CD11c and F4/80 and could find both DCs (CD11c⁺F4/80⁻, CD11c⁺F4/80⁺) and macrophages (CD11c⁻F4/80⁺) in the atherosclerotic intima (Figure S7A). Shown by FACS, atherosclerotic aorta had more CD45⁺ leukocytes and CD11c⁺MHC II^{hi} DCs than normal aorta, approximately 3~4 fold and 8~10 fold respectively (Figure S7B). When we analyzed the CD11c⁺MHC II^{hi} DCs, the CD11b⁺F4/80⁺ population was more dramatically increased throughout the aorta including the aortic sinus, aortic arch, and thoracic aorta (Figure S7B), but CD103⁺ DCs were also increased in atherosclerotic relative to normal aorta (Figure S7C).

To pursue the Flt3 dependence of the aortic cell subsets in disease, we generated atherosclerotic mice lacking Flt3 by crossbreeding *Ldlr*$^{-/-}$ to *Flt3*$^{-/-}$ mice. We confirmed that more than 99% of makers between *Flt3*$^{+/+}$*Ldlr*$^{-/-}$ and *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice were identical in microsatellite genotyping for C57BL/6 genetic background (data not shown). *Flt3*$^{+/+}$*Ldlr*$^{-/-}$ and *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice were fed a high-fat diet for 12 weeks, and we checked the aortic DC populations. The CD11b⁻CD103⁺ DCs were again highly dependent on Flt3 in atherosclerotic mice (Figure 7A, arrow), whereas CD11b⁺CD103⁻ DCs were able to expand in the *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice (Figure 7A). This result shows that DCs in atherosclerotic aorta can be generated from a classical Flt3-dependent DC precursor.

To understand the role of classical DCs in the development of atherosclerosis, we measured the lesion size in the aortic sinus of *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice compared with *Flt3*$^{+/+}$*Ldlr*$^{-/-}$ mice. The average lesion size of *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice was increased by

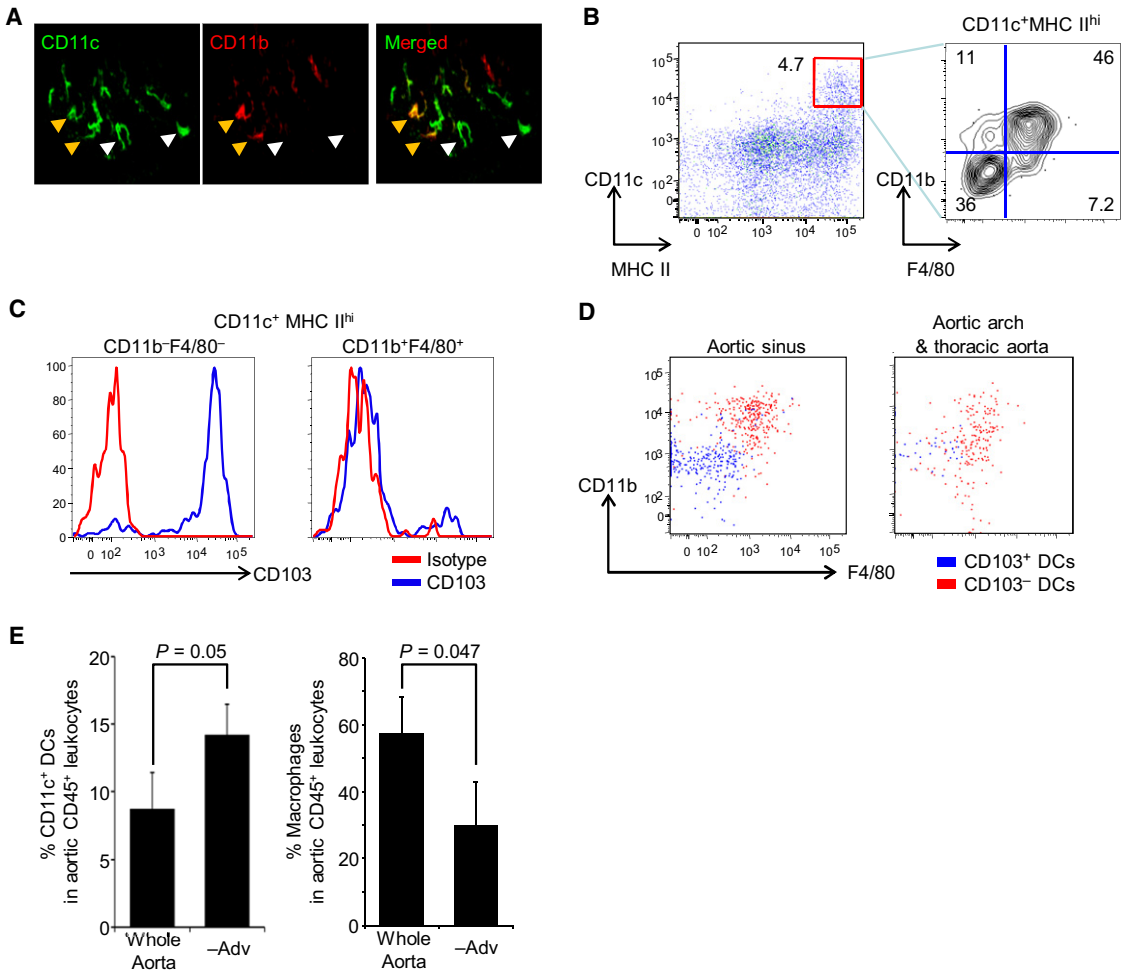

Figure 4. Presence of Two Major DC Subsets in Normal Aorta

(A) Double en face immunostaining for CD11c and CD11b in aortic intima to show that intimal $CD11c^+$ cells are either CD11b positive or negative.

(B) FACS analysis of $CD11c^+MHC\ II^{hi}$ $CD45^+$ aortic cells (red box) showing two major populations including $CD11b^+F4/80^+$ and $CD11b^-F4/80^-$ DCs.

(C) Selective expression of CD103 by the $CD11b^-$ $F4/80^-$ DC subset.

(D) Distribution of $CD103^+$ DCs (blue) in separately analyzed cells from aortic sinus and the rest of the aorta including aortic arch and thoracic aorta.

(E) Enrichment of the frequency of DCs by removal of adventitia (Adv) without altering the numbers of the DC subsets. Error bars refer to standard deviation. Each figure is representative of at least three experiments.

69% compared with $Flt3^{+/+}Ldlr^{-/-}$ mice (Figure 7B). In the aortic arch, the average lesion size of $Flt3^{-/-}Ldlr^{-/-}$ mice was also increased compared with $Flt3^{+/+}Ldlr^{-/-}$ mice (Figure 7C); the increase was smaller than in the aortic sinus, presumably because the aorta naturally has a smaller representation of Flt3-dependent DCs than the aortic sinus as shown above. The plasma lipid profiles were not different between $Flt3^{-/-}Ldlr^{-/-}$ and $Flt3^{+/+}Ldlr^{-/-}$ mice (Figure S7D), suggesting that the Flt3-dependent DCs were operating to reduce inflammation and immunity, not lipid metabolism. To understand the proatherogenic events in $Flt3^{-/-}Ldlr^{-/-}$ mice, we analyzed T cells in the atherosclerotic mice. Although total T cell numbers were not altered, the percentages of Treg cells in the aorta were markedly reduced in $Flt3^{-/-}Ldlr^{-/-}$ mice compared with $Flt3^{+/+}Ldlr^{-/-}$ mice (Figure 7D). To determine whether the lack of Treg cells was associated with more inflammation, we measured the expression of mRNA for the inflammatory cytokines IFN-γ and TNF-α in the aorta itself, and both were markedly increased in $Flt3^{-/-}Ldlr^{-/-}$ mice than controls (Figure 7E). These results indicate that Flt3-dependent classical DCs have an atheroprotective effect, possibly through increasing the regulatory T cell population.

DISCUSSION

Although macrophages in the aorta and large vessels have been the object of longstanding and considerable research (Woollard and Geissmann, 2010), studies on aortic DCs are more recent and less numerous (reviewed in Cybulsky and Jongstra-Bilen, 2010). Here, we compared DCs and macrophages side by side in the mouse aorta, whereas prior work has focused on one cell type or the other. Our first emphasis was to determine whether basic functional distinctions that have been evident in lymphoid and other nonlymphoid tissues were also evident in

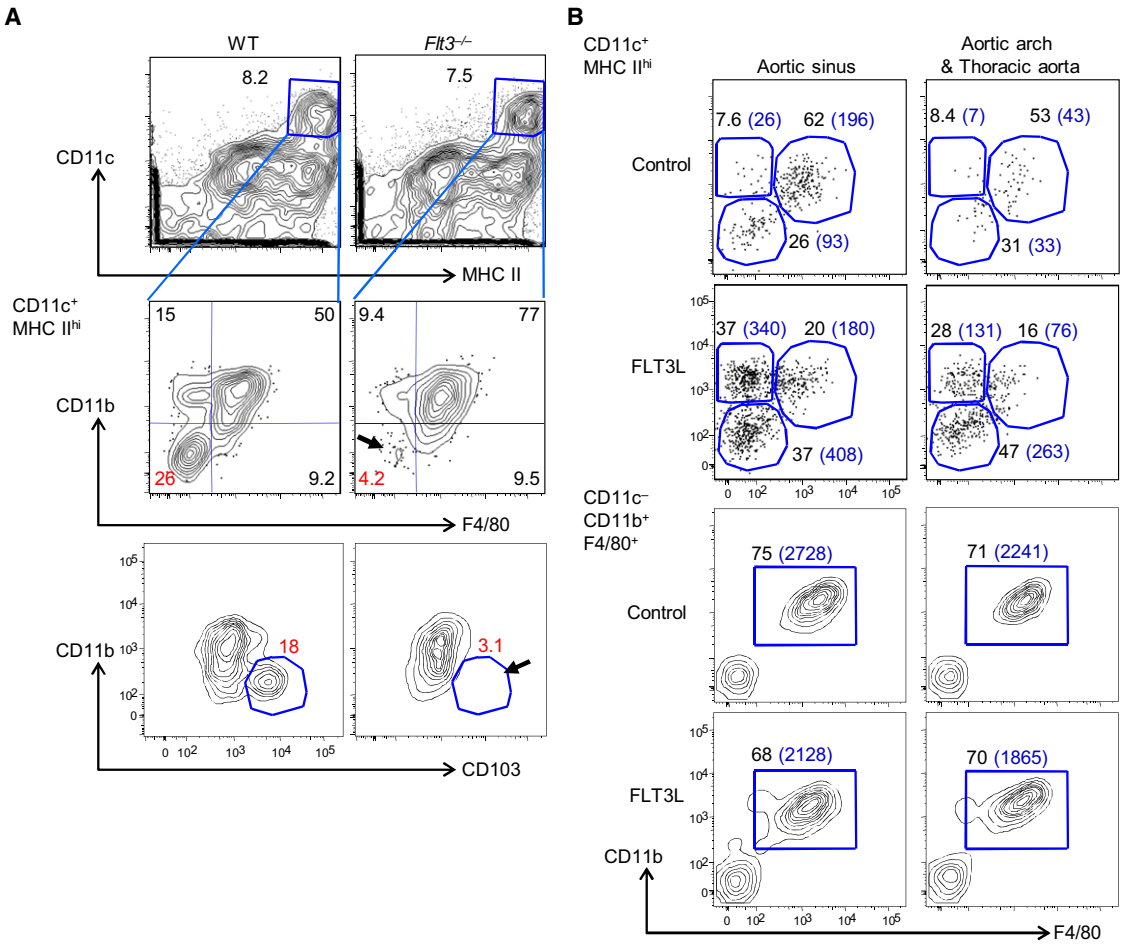

Figure 5. Dependence of CD11b$^-$F4/80$^-$CD103$^+$ DCs on the Flt3-Flt3L Pathway

(A) Selective decrease of CD11b$^-$F4/80$^-$CD103$^+$ DCs in Flt3-deficient mice (arrows).

(B) Expansion of F4/80$^-$ DCs by Flt3L treatment for 9 consecutive days, including a CD11b$^+$F4/80$^-$ population that was a scant population in steady state aorta. The expansions of these cells by Flt3L treatment were occurred in overall aorta including aortic sinus, aortic arch and descending aorta. Each figure is representative of at least three experiments.

the aorta. In fact, macrophages from the aorta were actively phagocytic but poorly immune stimulatory, whereas DCs were poorly phagocytic but strongly immunostimulatory. DCs were MHC II^{hi} and most macrophages MHC II^{lo}, but a subset of macrophages did express MHC II. The latter population was also inactive in stimulating the MLR, which is a test for the capacity of antigen-presenting cells to initiate immunity from many T cell clones in the polyclonal repertoire. It is possible that the aortic macrophages, many of which are in the adventitia, include monocytes capable of giving rise to either macrophages or monocyte-derived DCs. Nonetheless functionally distinct macrophages and DCs are found in the aorta as is the case with most tissues.

Aortic macrophages, which had low CD11c expression but high F4/80 and CD11b, were surprisingly localized to the adventitia of the aorta in steady state, except in the case of the aortic valve and sinus where macrophages were also noted in the intima. In contrast, DCs were CD11c$^+$MHC II^{hi} and were the dominant cell in the intima. As summarized in the introduction, prior emphasis has been on the monocyte as the principal cell in the aortic intima, but in the steady state, we found that CD11c$^+$MHC II^{hi} DCs dominated.

To further characterize the intimal cells as DCs, we examined their developmental origin. Surprisingly, both major pathways of DC development, termed classical and monocyte-derived DCs (Mo-DCs), were juxtaposed in the intima. The classical pathway is marked by CD103 and CD135 but lacks CX_3CR1, CD11b, and F4/80. It is dependent on Flt3-Flt3L for its development. The Mo-DC pathway lacks CD103 but expresses CX_3CR1, CD11b, F4/80, CD14, and DC-SIGN. It is dependent upon M-CSF for its development. Both types of DCs, after isolation from mice, function as classical mature DCs, lacking phagocytic activity but exerting strong stimulation of T cells (Steinman and Cohn, 1974; Steinman and Witmer, 1978).

Both types of DCs also were increased in experimental atherosclerosis, although the Mo-DCs increased to a greater extent. Recently, we and others found that Mo-DCs do exist in lymph nodes in the steady state but increase markedly upon microbial

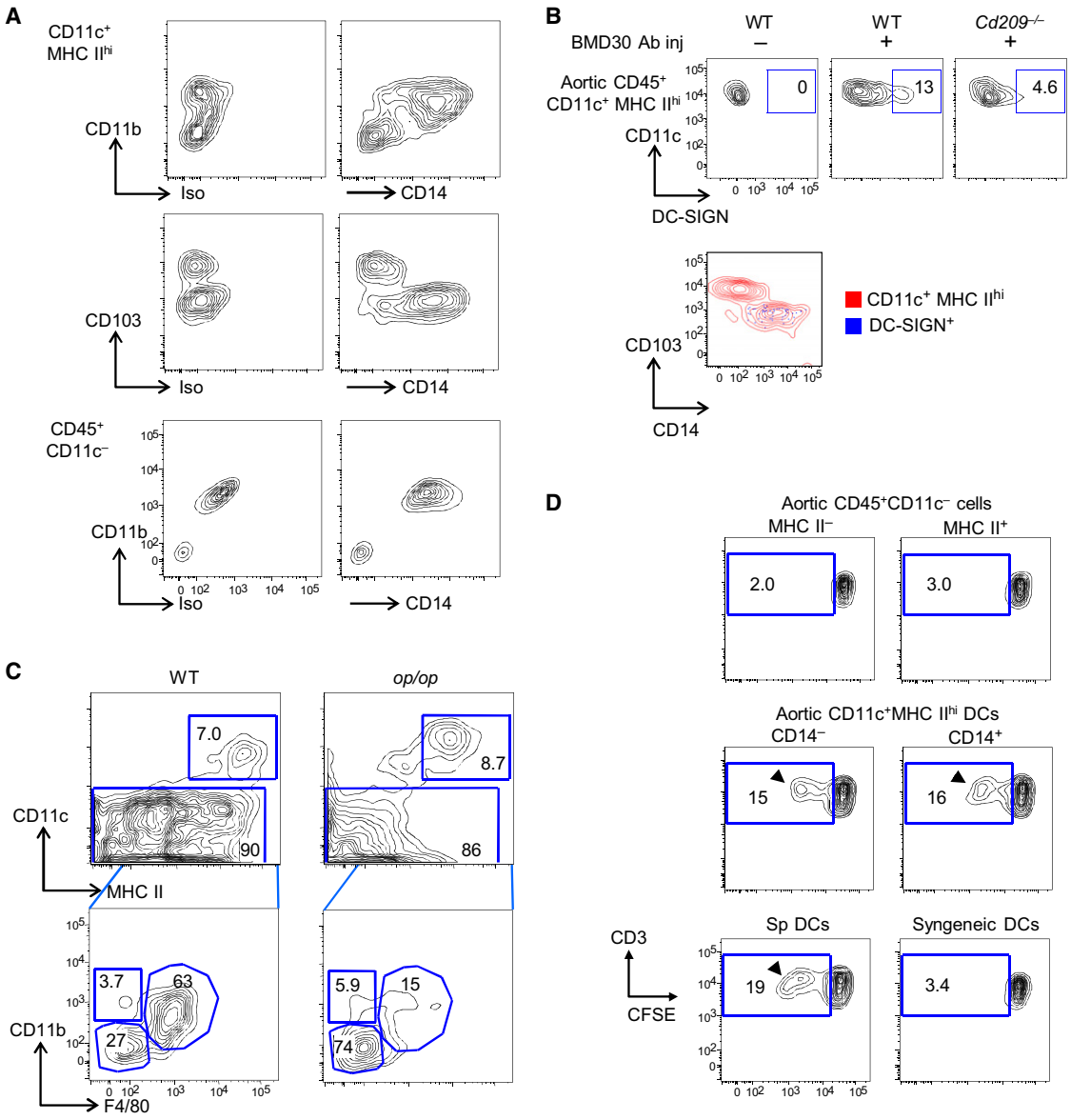

Figure 6. Dependence of CD11b$^+$F4/80$^+$CD103$^-$ DCs on the M-CSF and Monocyte Pathway

(A) The expression of CD14 on the CD11b$^+$CD103$^-$ subset of CD11c$^+$MHC II^{hi} DCs. As positive control (lower row), the aortic macrophages (CD11c$^-$CD11b$^+$F4/80$^+$) were stained for CD14.

(B) Labeling of the CD11b$^+$CD14$^+$ subset of DCs by DC-SIGN (blue dots). DC-SIGN antibody was injected i.v. for 12 hr (10 μg of Alexa647 labeled BMD30 clone).

(C) Depletion of CD11b$^+$F4/80$^+$ population in CD11c$^+$MHC II^{hi} DCs from the aorta of *op/op* mouse. Each figure is representative of at least three experiments.

(D) Stimulation of MLR by CD14$^+$ DCs (C57BL/6) mixed with CFSE-labeled T cells. The arrow indicates CFSE-dilution of CD3$^+$ T cells (BALB/c). Syngeneic DCs were from the spleen of BALB/C mouse. Each figure is representative of two or three experiments.

infection (Cheong et al., 2010a) or some conditions of chronic inflammation such as colitis (Siddiqui et al., 2010). However, there are no decisive genetic tools yet to selectively ablate Mo-DCs transiently or constitutively. Genetic models currently available such as CD11c-DTR, CD11b-DTR, and LysM-Cre × iDTR also affect monocyte and macrophage populations.

With these developmental criteria, we could perform experiments that provided unexpected results on the role of classical DCs in atherosclerosis. When Flt3 was genetically deleted from *Ldlr*$^{-/-}$ atherosclerosis-prone mice, the corresponding DC subset was selectively lost from the aorta, and atherosclerosis was aggravated. This indicates that Flt3$^+$ DCs protect against this inflammatory disease. In contrast M-CSF is known to be a proatherogenic molecule (Chitu and Stanley, 2006; Rajavashisth et al., 1998), although it is not yet clear whether this effect is entirely from monocytes and macrophages or also includes Mo-DCs.

A prior study immunized *Ldlr*$^{-/-}$ mice with bone marrow-derived DCs that had taken up oxidized low-density lipoproteins and observed a protective effect of such "DC vaccines"

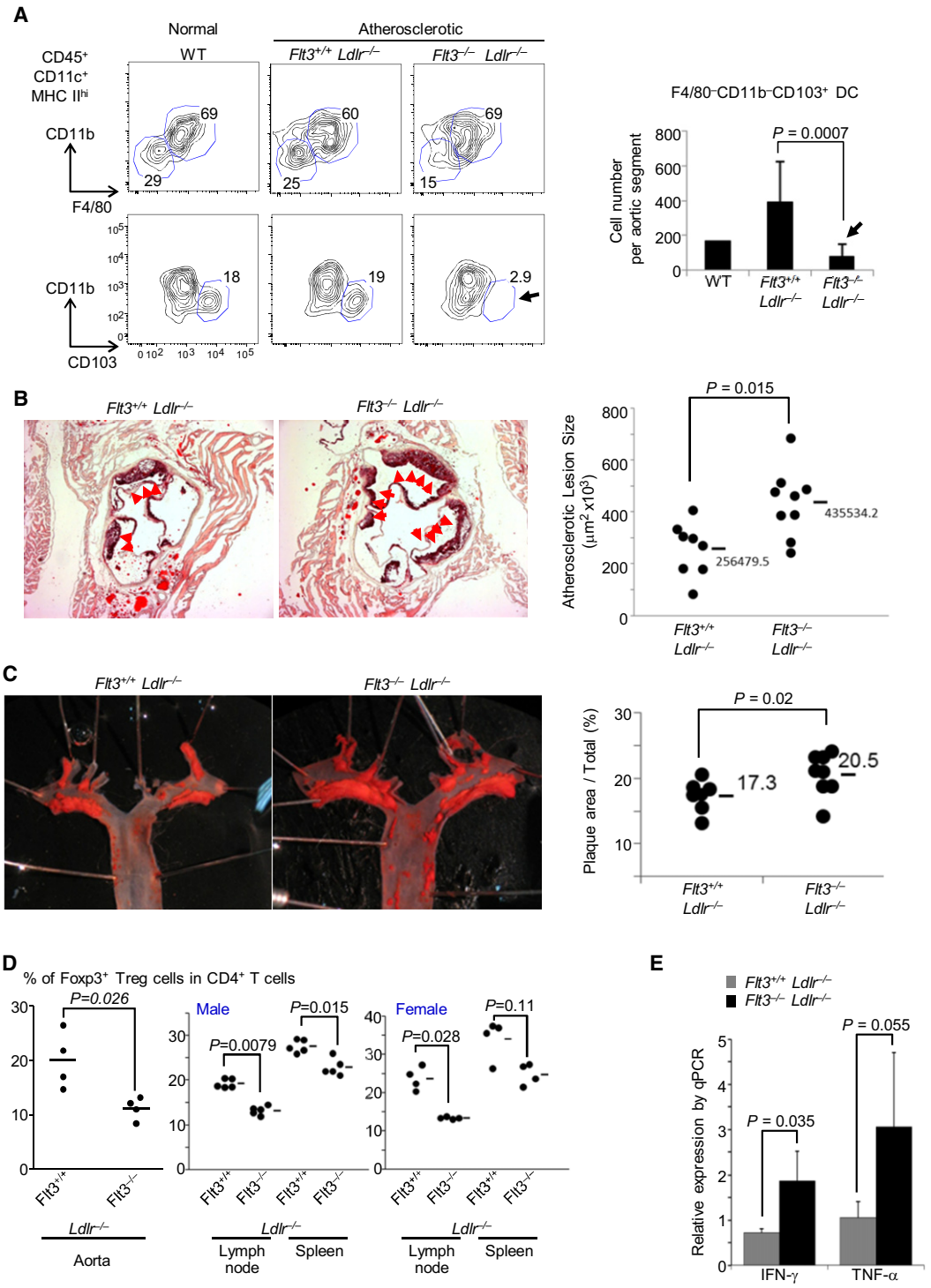

Figure 7. Atheroprotective Effects of Flt3-Dependent, CD103⁺ DCs

(A) Flt3 dependency of CD11b⁻F4/80⁻CD103⁺ DCs (arrows) during atherosclerosis of *Flt3*$^{+/+}$*Ldlr*$^{-/-}$ and *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice fed a high-fat diet for 12 weeks.

(B) Increased lipid deposits by oil red O staining of atherosclerotic lesion (illustrative stains on left, measurements of individual mice on right) formed in the aortic sinus of *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice (n = 9) relative to *Flt3*$^{+/+}$*Ldlr*$^{-/-}$ mice (n = 8) after a high-fat diet for 12 weeks.

(C) As in (B), but aortic en face Oil red O staining.

(D) The percentage of Treg cells (CD3⁺CD4⁺Foxp3⁺) in the leukocytes of aorta, lymph node and spleen in the atherosclerotic *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ compared to *Flt3*$^{+/+}$*Ldlr*$^{-/-}$ mice. For aorta, only male mice were used.

(E) Increased expression of mRNA for IFN-γ and TNF-α in the atherosclerotic aortas of *Flt3*$^{-/-}$*Ldlr*$^{-/-}$ mice. All mice were fed a high-fat diet for 12 weeks. Error bars refer to standard deviation.

(Habets et al., 2010). However, research on the effect of DC alterations in situ in atherosclerosis has not provided a clear picture. When the life span of DCs was prolonged with CD11c-hBcl2 transgenic mice, plasma lipids were decreased, whereas DC ablation with CD11c-DTR transgenic mice increased blood lipids (Gautier et al., 2009). Cybulsky and his colleagues recently demonstrated that DC ablation in CD11c-DTR transgenic mice reduced intimal lipid accumulation, suggesting a proatherogenic role in early disease (Cybulsky and Jongstra-Bilen, 2010; Paulson et al., 2010). A difficulty with prior work is that CD11c expression in atherosclerotic aorta is not restricted to DCs but expressed by macrophage foam cells (Cho et al., 2007), which we confirmed by noting $CD11c^+$ but MHC II^- foam cells in atherosclerotic mouse aorta (data not shown). Moreover, it is not possible to dissect the function of DC subtypes with CD11c-DTR mice given that CD11c is expressed on both major DC types in the aorta. Currently, the developmental approaches used here seem to be more discriminating for identifying the functions of DCs in situ, as illustrated by the atheroprotective effects we found in mice deficient for Flt3.

In addition to monocyte-derived DCs, the aorta has Flt3-Flt3L-dependent classical DCs, from the $CD103^+CD11b^-F4/80^-$ population. In steady state, these DCs are mainly localized in the aortic sinus. But, in atherosclerosis, we could see the increase of these DCs in the overall aorta. It has been known that classical DCs can induce a tolerance in steady state, and constitutive DC ablation can develop autoimmune disease (Ohnmacht et al., 2009). For example, the $CD103^+$ $CD11b^-$ subset of DCs in the intestinal mucosa is able to induce $Foxp3^+$ regulatory or immune-suppressive $CD4^+$ T cells (Coombes et al., 2007; Sun et al., 2007a), and these T cells have been implicated in the reduction of atherosclerosis (Ait-Oufella et al., 2006). When Flt3 was genetically deleted from $Ldlr^{-/-}$ atherosclerosis-prone mice, the corresponding DC subset was selectively lost from the aorta, and atherosclerosis was aggravated considerably. $Foxp3^+$ T cells were decreased in Flt3-deficient atherosclerotic mice, and others have reported that increased Treg cells in lymphoid tissues are associated with reduced atherosclerosis (Ait-Oufella et al., 2006; Mor et al., 2007; Hermansson et al., 2011). It is known that the numbers of $Flt3^+$ DCs and Treg cells are reciprocally related (Darrasse-Jèze et al., 2009). While revising our manuscript, a subset of $CD11b^+$DCs expressing CCL17-EGFP was reported to restrain the homeostasis of Treg cells, thereby promoting atherosclerosis (Weber et al., 2011) in contrast to the expansion of Treg cells by cDCs reported here. The developmental origin and function of CCL17-$EGFP^+$ $CD11b^+$DCs also remains to be explored. Here, we extended prior work by showing that the decrease in Treg cells in $Flt3^{-/-}Ldlr^{-/-}$ mice is also associated with higher expressions of mRNA for two inflammatory cytokines tested, IFN-γ and TNF-α, in the aorta itself. These findings provide a more precise developmental and functional picture of the cell types in the aorta that is very different from prior analyses that were based on cell surface markers primarily and support the view that "the immune response in atherosclerosis is a double edged sword," with one subset of DCs providing a protective edge (Hansson and Libby, 2006).

EXPERIMENTAL PROCEDURES

Mice

$Ldlr^{-/-}$, CD11b-DTR, *op/op*, and $Cx_3cr1^{gfp/+}$ mice were obtained from Jackson laboratory. $Flt3^{-/-}$ mice (Matthews et al., 1991) (I. Lemischka, Mount Sinai School of Medicine) were a generous gift of M. Nussenzweig. Langerin-EGFP mice were a generous gift of B. Malissen. *DC-SIGN/Cd209a*$^{-/-}$ mice were from the Consortium for Functional Glycomics (Scripps Res. Inst., La Jolla, CA, USA). We used C57BL/6 (CD45.1 or CD45.2) mice and BALB/C mice from Taconic. Mice were maintained under specific pathogen-free conditions and fed ad libitum a normal mouse diet unless indicated. All mice were used in accordance with guidelines of the Institutional Animal Care and Use Committee, the Rockefeller University.

Antibodies and Reagents

mAb to cell surface markers CD14 and CD103 were from eBioscience and all others from BioLegend. Anti-Foxp3 antibody and Foxp3 staining buffer set were from e-Bioscience. Dynalbeads and Tyramide signal amplification (TSA) kit were from Invitrogen. Collagenase I, collagenase XI, hyaluronidase, and DNaseI were from Sigma Aldrich. Mouse Flt3 ligand was produced in house as described (Darrasse-Jèze et al., 2009).

Aortic Single-Cell Preparations and Flow Cytometric Analysis

Aortic single cells were prepared in accordance with a previous method (Choi et al., 2009) with minor modification. In brief, after careful removal of the perivascular fat and cardiac muscle tissues, with microscissors under a dissecting microscope, single-cell suspensions from aortic segments, including aortic sinus, or aortic arch and thoracic aorta, were prepared by incubation with an enzyme mixture containing 675 U/ml collagenase I, 187.5 U/ml collagenase XI, 90 U/ml hyaluronidase, and 90 U/ml DNase in Hank's balanced salt solution with calcium and magnesium for 75 min at 37°C with gentle shaking. Splenic single-cell suspensions were prepared by incubation with 400 U/ml collagenase D at 37°C for 30 min. After blocking Fc receptors with culture supernatant from the 2.4G2 hybridoma, the cells were stained with the indicated fluorochrome-conjugated antibodies. We used LSRII instrument (BD) and analyzed flow cytometric data with FlowJo (Tree Star Inc).

Analysis of Phagocytosis

Aortic single cells were incubated with 0.00134% of 0.50μm Fluoresbrite YG Microspheres (Polysciences) for 70 min at 37°C. The cells were washed with cold PBS twice. Then the cells were labeled with mAbs to CD45, CD11c, MHC II, and CD11b and analyzed with ImageStream (Amnis) or flow cytometry (BD LSRII).

En Face Immunostaining and Confocal Microscopic Analysis

The aorta was perfused with cold 4% paraformaldehyde in PBS via the left ventricle. After removing perivascular tissues, segments of aortic sinus, aortic arch, and thoracic aorta were opened longitudinally and further fixed in 4% paraformaldehyde at 4°C for 30 min. After permeabilization with 0.2% Triton X-100, staining for CD11c, CD11b, and F4/80 was performed using Alexa 488 and Alexa 568 TSA kit (Invitrogen) following the manufacturer's protocol. After staining, the aortic segments were mounted on glass slides with aqueous mounting medium. The confocal images of en face immunostaining were obtained with a Zeiss 510 Meta inverted laser scanning microscope. All confocal sections were taken along the Z axis for visualizing all processes from each DC.

Bone Marrow Chimeras

Mixtures (50:50) of marrow from $CD45.2^+$ LysM-Cre × iDTR and $CD45.1^+$ WT mice were intravenously (i.v.) injected to lethally irradiated (5.5 Gy, 3 hr apart) CD45.1 mice. After 6 weeks, DT (25 ng/g weight) was i.v. injected and the aortic leukocyte populations were analyzed.

Mixed Leukocyte Reactions

The aortic DC populations ($CD45^+CD11c^+$MHC II^{hi}) and macrophages ($CD45^+$ $CD11c^-CD11b^+$) were isolated from single-cell suspensions of aorta with FACS sorting (BD FACS Vantage or FACSAria). Allogeneic T cells were isolated from spleens of BALB/C mice by excluding $B220^+$, $F4/80^+$, $CD49b^+$, and $I\text{-}Ab^+$

cells with anti-rat IgG Dynalbeads (Invitrogen). T cells were added to round-bottom microtest wells at 25,000/well and mixed with isolated DCs at a DC:T cell ratio of 1:2 and 1:10. After 5 days, the proliferation of T cells was evaluated by CFSE dilution on the FACS.

Assessment of Atherosclerosis

In mouse models of atherosclerosis, the atherosclerosis-prone areas are the aortic sinus, lesser curvature of aortic arch, and the openings of arterial branches. The lesions appear in the aortic sinus, and as the diet is prolonged, the atherosclerotic lesions appear in other parts of aorta including the aortic arch and opening of arterial branches. Here, we analyzed atherosclerosis in two different atherosclerosis-prone areas the aortic sinus and aortic arch. *Ldlr*$^{-/-}$ mice fed a high-fat diet with 0.15% cholesterol and 20% fat but no sodium cholate (Harlan) for 12 or 16 wks were euthanized with CO_2 inhalation. The heart and aorta were perfused with cold 4% paraformaldehyde through the left ventricle. After additional fixation, the heart was embedded in OCT and frozen. Aorta was dissected from the proximal ascending aorta to thoracic aorta, and adventitial fat was removed. For en face analysis, aorta was split longitudinally, pinned onto flat black silicone plates, and stained with oil red O for 4 hr, washed with PBS briefly, and digitally photographed at a fixed magnification. Total aortic areas and lesion areas were calculated with Axio Vision (Carl Zeiss, German). For analyzing aortic sinus plaque lesions, cryosections were stained with oil red O overnight and digitized.

Quantitative Real-Time PCR

Total aortic RNA was isolated with Trizol reagent (Invitrogen), and cDNA was synthesized with the Superscript III First-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR was performed using a 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) with mouse IFN-γ, TNF-α, and GAPDH primers and probes (Applied Biosystems, Assay-on-DemandTM). The absolute number of gene copies was normalized with GAPDH and standardized using a sample standard curve.

Statistical Analysis

Results were analyzed with Mann-Whitney U test or unpaired Student's t test with two-tailed distributions (GraphPad Instat) when two groups were compared, or Kruskal-Wallis test followed by Mann-Whitney U test, for comparisons of more groups relative to the control group.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.immuni.2011.09.014.

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