Conventional Dendritic Cells at the Crossroads Between Immunity and Cholesterol Homeostasis in Atherosclerosis

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Background—Immunoinflammatory mechanisms are implicated in the atherogenic process. The polarization of the immune response and the nature of the immune cells involved, however, are major determinants of the net effect, which may be either proatherogenic or antiatherogenic. Dendritic cells (DCs) are central to the regulation of immunity, the polarization of the immune response, and the induction of tolerance to antigens. The potential role of DCs in atherosclerosis, however, remains to be defined.

Methods and Results—We created a mouse model in which the lifespan and immunogenicity of conventional DCs are enhanced by specific overexpression of the antiapoptotic gene hBcl-2 under the control of the CD11c promoter. When studied in either low-density lipoprotein receptor–deficient or apolipoprotein E–deficient backgrounds, DC-hBcl2 mice exhibited an expanded DC population associated with enhanced T-cell activation, a T-helper 1 and T-helper 17 cytokine expression profile, and elevated production of T-helper 1–driven IgG2c autoantibodies directed against oxidation-specific epitopes. This proatherogenic signature, however, was not associated with acceleration of atherosclerotic plaque progression, because expansion of the DC population was unexpectedly associated with an atheroprotective decrease in plasma cholesterol levels. Conversely, depletion of DCs in hyperlipidemic CD11c–diphtheria toxin receptor/apolipoprotein E–deficient transgenic mice resulted in enhanced cholesterolemia, thereby arguing for a close relationship between the DC population and plasma cholesterol levels.

Conclusions—Considered together, the present data reveal that conventional DCs are central to the atherosclerotic process, because they are directly implicated in both cholesterol homeostasis and the immune response. (Circulation. 2009;119: 2367-2375.)

Key Words: atherosclerosis ■ immune system ■ homeostasis ■ dendritic cells ■ lymphocytes

Dendritic cells (DCs) are the most potent antigen-presenting cells. Indeed, DCs possess a markedly elevated capacity to stimulate T cells, B cells, and natural killer T cells and to drive T-cell differentiation along both T-helper 1 (Th1) and T-helper 2 (Th2) pathways. Moreover, DCs are known to favor tolerance to antigens, possibly via the generation of regulatory T cells. As major regulators of immune responses and T-cell polarization, DCs are potentially key players in chronic inflammatory diseases such as atherosclerosis. Indeed, available evidence suggests that immune responses are directly implicated in the pathogenesis of atherosclerosis. Although the presence of DCs has been reported in atherosclerotic plaques, no mechanistic insight into the potential central immunoregulatory role of DCs in the immunoinflammatory dimension of atherosclerosis has been provided in atherosclerosis-prone mice. Modulation of the capacity of DCs to induce an immune response may facilitate evaluation of their impact on the pathogenesis of atherosclerosis. Indeed, enhancement of the lifespan of DCs has been reported to increase their immunogenicity in mice. In this context, it is especially relevant that Bcl-2 has been shown to be a major regulator of DC lifespan and immunogenicity. We therefore developed a mouse model in which DC lifespan and immunogenicity are enhanced by overexpression of human Bcl-2 (hBcl-2) under the control of the DC-specific CD11c promoter. This experimental approach allowed us to modulate the half-life and thus the immunogenicity of DCs in vivo with a view to evaluate their impact on the immune response during atherosclerosis.
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Methods
Transgenic mice expressing hBcl-2 under the murine CD11c promoter were described previously. All procedures (bone marrow transplantation, plasma lipid analyses, chimerism, quantification of atherosclerotic plaques, immunohistochemistry, quantification of autoantibodies, analysis of gene expression by quantitative polymerase chain reaction, flow cytometry, cytokine assays, and generation of bone marrow–derived DCs) were performed as described previously and are described in the online-only Data Supplement, along with details about the animal model.

Statistical Analysis
The statistical significance of the differences between groups was evaluated with the unpaired or paired 2-tailed Student t test. \( P < 0.05 \) was considered significant. Values are expressed as mean \( \pm \) SEM.

Results
Characterization of DC-hBcl2 Mice
Because CD11c is differentially expressed by DC subpopulations, and to gain insight into the specificity of transgene expression, we assessed the expression of hBcl-2 in DC subpopulations and in different types of leukocytes (online-only Data Supplement Figure I). Flow cytometric analysis revealed that splenic conventional DCs (CD11c\(^{+}\), MHCII\(^{+}\)), comprising CD11b\(^{-}\) DCs (CD11c\(^{+}\), CD11b\(^{-}\)) and CD8\(^{+}\) DCs (CD11c\(^{+}\), CD8\(^{+}\)), expressed hBcl-2, whereas plasmacytoid DCs (CD11c\(^{hi}\), PDCA-1\(^{+}\)) did not. Equally, we demonstrated that splenic B cells, T cells, and macrophages did not express hBcl-2. Another population of leukocytes known to express CD11c, bronchoalveolar macrophages (CD11c\(^{-}\), F4/80\(^{+}\)), did not express hBcl-2, whereas DC-hBcl-2 in the DC-hBcl-2 mice in the present study (online-only Data Supplement Figure I). This finding may be explained by the nature of the CD11c promoter used, which is a minimal promoter previously described to drive expression only in DCs with high endogenous expression of CD11c. In our model, monocyes (CD11b\(^{-}\), F4/80\(^{+}\)) did not express hBcl-2, and therefore, monocye count was similar in control apolipoprotein E–deficient (Apoe\(^{-/-}\)) mice and DC-hBcl-2 Apoe\(^{-/-}\) mice (online-only Data Supplement Figure II).

hBcl-2 Overexpression in DCs Enhances Their Lifespan and Immunogenicity
DCs generated from bone marrow cells of DC-hBcl-2 mice expressed hBcl-2 protein as expected (online-only Data Supplement Figure IIIA) and displayed enhanced resistance to apoptotic stress (online-only Data Supplement Figure IIIB). Such enhanced survival impacted the relative number of DCs in vivo. Indeed, the DC population was enriched in spleens from DC-hBcl-2 mice (online-only Data Supplement Figure IIIC; \( P < 0.05 \)). Then, because DCs may control lymphocyte homeostasis, we assessed T-cell activation in splenocytes of DC-hBcl-2 and control mice at the basal state. The data revealed that activated T cells, CD3\(^{+}\) and CD4\(^{+}\) cells expressing the activation marker CD69, were significantly increased in DC-hBcl-2 mice compared with controls (online-only Data Supplement Figure IID; \( P < 0.01 \) and \( P < 0.001 \), respectively), whereas expression of CD25 by CD4\(^{+}\) T cells was similar in both groups in the basal state (online-only Data Supplement Figure IIID). These data are consistent with an enhanced immunogenicity of DCs in DC-hBcl-2 mice fed a chow diet. In this regard, it is relevant that on a nonlethal lipopolysaccharide challenge, we reported that DC-hBcl-2 mice equally exhibited significant elevation in the DC population, as well as in activation of T and B cells, compared with their littermate controls. Collectively, hBcl-2 overexpression in DCs prolonged their lifespan, led to a significant increase in the DC population, and enhanced T-cell activation in vivo.

Effect of Enhanced DC Lifespan and Immunogenicity on T-Cell Activation in Ldl-r\(^{-/-}\) Mice
To evaluate whether DC lifespan and immunogenicity impact both immunity and atherogenesis, irradiated female low-density lipoprotein (LDL) receptor–deficient (Ldl-r\(^{-/-}\)) mice were reconstituted with bone marrow cells from DC-hBcl-2 mice or wild-type (WT) littermates. After 4 weeks of recovery, mice were switched to a Western diet for 12 weeks. The efficiency of transplantation was established by the detection of \(<5\%\) of Ldl-r knockout alleles in bone marrow cells from these mice (online-only Data Supplement Figure IV), thereby indicating a chimerism in the range of 95% to 100%.

We first evaluated the impact of enhancement of DC lifespan on the DC population itself and on T-cell activation and Th1 polarization. DCs were enriched in the spleens of DC-hBcl-2→Ldl-r\(^{-/-}\) compared with wt→Ldl-r\(^{-/-}\) mice (+56%, \( P < 0.01 \); Figure 1A). Analysis of splenic T cells revealed an elevation in the proportion of both CD3\(^{+}\) and CD4\(^{+}\) T cells expressing the activation marker CD69 (\( P < 0.0001 \) for each) and of CD4\(^{+}\) T cells expressing CD25 (+25%, \( P < 0.01 \)) in DC-hBcl-2→Ldl-r\(^{-/-}\) compared with controls (Figure 1B). Concomitantly, an increment of 20% in the percentage of CD44-expressing CD4\(^{+}\) memory T cells was observed in DC-hBcl-2→Ldl-r\(^{-/-}\) compared with wt→Ldl-r\(^{-/-}\) mice (\( P < 0.0001 \); Figure 1C). We next quantified the mRNA expression of key mediators of DC function, as well as T-cell responses and polarization in the spleen. Analysis of the expression of genes characteristic of DC function revealed a significant increase in the mRNA of interleukin (IL)-12p40, IL-23p19, and IL-15 in DC-hBcl-2→Ldl-r\(^{-/-}\) mice, whereas expression of the IL-12p35 and IL-18 genes was unchanged (Figure 1D). This was associated with enhanced expression of interferon (IFN)-\(\gamma\) and TIM-3 (T-cell immunoglobulin- and mucin-containing molecule, a transcription factor promoting Th1 development), together with unchanged levels of GATA3 mRNA (a transcription factor promoting Th2 development) in DC-hBcl-2→Ldl-r\(^{-/-}\) mice (Figure 1E). Expression levels of classic inflammatory genes revealed elevated levels of IL-1\(\beta\) mRNA but similar levels of CD40L and tumor necrosis factor-\(\alpha\) mRNAs in the spleens of DC-hBcl-2→Ldl-r\(^{-/-}\) mice compared with controls (Figure 1E). These findings support the contention that an enhanced DC lifespan leads to elevation in DC immunogenicity and increased T-cell activation with polarization toward a Th1 profile.
Effect of Enhanced DC Lifespan and Immunogenicity on Regulatory T Cells in Ldl-r<sup>−/−</sup> Mice

Natural regulatory T cells (Treg) exhibit marked antiatherogenic properties<sup>18</sup> as a consequence of their ability to counteract both Th1- and Th2-mediated immune responses. Because DCs might influence the content and function of regulatory T cells, we evaluated whether the Treg population was modified in DC-hBcl-2→Ldl-r<sup>−/−</sup> mice compared with wt→Ldl-r<sup>−/−</sup> mice. As shown in Figure 2A, flow cytometric analysis revealed that spleen CD4<sup>+</sup> Foxp3<sup>+</sup> and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells were similar in both groups, thereby arguing for the absence of an altered natural Treg population. We next quantified the expression of key markers of Treg cell population and function in the spleens of both groups of mice. Real-time quantitative polymerase chain reaction confirmed the unaltered Foxp3 expression consistent with the absence of elevation in the natural Treg population. In this context, it was relevant that expression levels of transforming growth factor-β (TGFβ) and CTLA-4 (cytotoxic T-lymphocyte–associated protein 4) were similar in splenic cells of both DC-hBcl-2→Ldl-r<sup>−/−</sup> and wt→Ldl-r<sup>−/−</sup> mice (Figure 2B). By contrast, a marked 7-fold increment in IL-10 mRNA expression was observed in DC-hBcl-2→Ldl-r<sup>−/−</sup> mice (Figure 2B; P<0.01). Overall, these data indicate that Treg cells are not markedly altered in DC-hBcl-2→Ldl-r<sup>−/−</sup> compared with wt→Ldl-r<sup>−/−</sup> mice.

Effect of Enhanced DC Lifespan and Immunogenicity on B-Cell Activation and Circulating Levels of Autoantibodies Against Oxidation-Specific Epitopes in Ldl-r<sup>−/−</sup> Mice

Several recent studies have emphasized the protective role of B lymphocytes in atherosclerosis.<sup>15,19,20</sup> These findings led us to question whether the increment in the DC population in our mouse model might affect levels of antibodies directed against oxidation-specific epitopes, the titer of the atheroprotective EO6 antibody idiotype, and the polarization of the humoral response (Th2-driven IgG1 versus Th1-driven IgG2c/IgG3 isotype production). We first evaluated B-cell activation by measuring the proportion of B cells bearing the protective EO6 antibody idiotype, and the polarization of the IgG2c fraction of both anti-MDA-LDL and anti-oxidized LDL antibodies (2-fold; Figure 3B and 3C; P<0.0005 for each) in DC-hBcl-2→Ldl-r<sup>−/−</sup> compared with control mice, whereas levels of IgG1, IgG3, and IgM fractions were comparable between groups (Figure 3B and 3C). Moreover, titers of the EO6 antibody were markedly elevated in DC-hBcl-2→Ldl-r<sup>−/−</sup> compared with wt→Ldl-r<sup>−/−</sup> mice (Figure 3D; P<0.0005). In conclusion, the increment in IgG2c titer in DC-hBcl-2→Ldl-r<sup>−/−</sup> mice, which is characteristic of a Th1-driven immune response, is consistent
The progression of atherosclerosis was evaluated in DC-hBcl-2 transgenic and control mice on an Western diet (247.0 ± 17.1 mg/dL versus 170 ± 42 mg/dL, respectively; Table). Analysis of cholesterol distribution among plasma lipoprotein subclasses revealed that the lower total cholesterol level in DC-hBcl-2 compared with wt mice was due to a reduction in the abundance of VLDL and LDL subclasses (−59% and −57%, respectively), whereas the HDL fraction was decreased to a lesser degree (−33%; Figure 5A). Because a larger DC population in DC-Bcl-2 animals was associated with lower circulating cholesterol levels, thereby revealing that the role of DC is important in the setting of hypercholesterolemia, we sought to determine whether the opposite mechanism (ie, acute depletion of DCs) was associated with an enhanced degree of cholesterolemia. We took advantage of the DT receptor (DTR)/diphtheria toxin (DT)

Table. Body Weight and Lipid Parameters

<table>
<thead>
<tr>
<th>Study</th>
<th>Wild-Type</th>
<th>DC-hBcl-2</th>
<th>P</th>
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<tr>
<td>Bone marrow transplantation in Ldl-r−/− mice, n</td>
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<td>14</td>
<td></td>
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<tr>
<td>Weight, g</td>
<td>19.7 ± 1.1</td>
<td>19.6 ± 1.3</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>547 ± 61</td>
<td>426 ± 63</td>
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<tr>
<td>Free cholesterol, mg/dL</td>
<td>198 ± 45</td>
<td>149 ± 33</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>192 ± 48</td>
<td>170 ± 42</td>
<td>0.34</td>
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<td>n=10</td>
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<td>Weight, g</td>
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<td>20.9 ± 3.5</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
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<td>317 ± 18</td>
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</tr>
<tr>
<td>Free cholesterol, mg/dL</td>
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<td>102 ± 6</td>
<td>&lt;0.01</td>
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<td>Triglycerides, mg/dL</td>
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<td>Weight, g</td>
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<td>20.7 ± 0.3</td>
<td>0.12</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
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<td>509 ± 19</td>
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<td>Free cholesterol, mg/dL</td>
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<td>&lt;0.05</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>79 ± 17</td>
<td>97 ± 10</td>
<td>0.41</td>
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</table>

Values are expressed as mean ± SEM unless otherwise indicated.
T-cell populations were larger in CD25-expressing CD4 than in controls (Figure 6B; **P<0.01**). Incubation experiments were conducted in DC-hBcl-2 Apoe mice after 12 weeks of Western diet. B, DC-hBcl-2→Ldl-r-/+ mice were treated with PBS or diptheria toxin (DT) to induce DC depletion. Plasma total cholesterol was determined in both groups. *P<0.05.

**Effect of DC Lifespan and Immunogenicity on Immune Response in Apoe−/− Mice**

We next evaluated whether the impact of DC lifespan and immunogenicity on immunity that we documented in Ldl-r−/− mice, and more especially that which involved T-cell activation and B-cell responses, was equally manifest in an alternative atherosclerotic model (ie, Apoe−/− mice). We first showed that the DC population was significantly expanded in chow-fed 20-week-old DC-hBcl-2 Apoe−/− mice compared with Apoe−/− controls (Figure 6A; **P<0.05**). Flow cytometric analysis revealed that both the CD3− and CD4−-activated T-cell populations were larger in DC-hBcl-2 Apoe−/− mice than in controls (Figure 6B; **P<0.001** each), whereas the CD25-expressing CD4+ T-cell population was unaltered (Figure 6B). With regard to the B-cell compartment, no change was observed in CD86 expression by B cells in DC-hBcl-2 Apoe−/− mice compared with controls (Figure 6C). The main alteration in the antibody response observed on the Ldl-r−/− background (ie, elevation in circulating levels of both anti-malondialdehyde-LDL and anti-oxidized LDL IgG2c antibodies in DC-hBcl-2 mice) was equally observed on an Apoe−/− background (Figure 6D and 6E; **P<0.05**), whereas no statistically significant differences were observed for the other isotypes. Finally, levels of the E06 antibody were not affected in DC-hBcl-2 Apoe−/− mice compared with controls. To further evaluate whether changes in the polarization of the immune response observed in DC-hBcl-2→Ldl-r−/− were equally present in the Apoe−/− background, additional experiments were conducted in DC-hBcl-2 Apoe−/− and Apoe−/− mice fed a Western diet. We thus confirmed activation of key mediators of DC function and T-cell responses in the spleen of DC-hBcl-2 in this background (ie, IL-12p40 and IL-23p19 mRNAs; online-only Data Supplement Figure VA). In addition, we characterized the regulatory response in the Apoe−/− background and confirmed the absence of an effect on natural Treg (nTreg) as shown by the absence of major changes in the expression of key genes involved in nTreg function and development (CD25, GITR, ICOS, neuropilin-1, and Drosha; online-only Data Supplement Figure VB). Although a major increase in IL-10 mRNA levels was observed in spleens of DC-hBcl-2→Ldl-r−/− mice (Figure 2B), a trend for higher IL-10 mRNA levels in Western diet-fed DC-hBcl-2 Apoe−/− mice was detectable (online-only Data Supplement Figure VA). However, in assays of restimulated splenocytes, CD4+ T cells from DC-hBcl-2 mice produced significantly more IL-10 than control CD4+ T cells, whereas DCs from DC-hBcl-2 mice produced less IL-10 than controls (Figure 7A and 7B). Such data suggest that changes in DC function and population are associated with increased production of IL-10 by CD4+ T cells, most likely T regulatory type 1 (Tr-1) T cells. Finally, we observed a 4.2-fold increase in the percentage of CD4+ IL-17+ cells compared with control Apoe−/− mice in restimulated splenocytes (Figure 7C). Moreover, intracellular staining for IFN-γ in CD4+ T cells (Figure 7D and 7E) confirmed the Th1 signature, which indicates that
A new increase in the activity of the Th17 pathway and a potential
35.3 21.7 versus 247.4 /H11003 /H11006 groups (Figure 8B; 184.1
103 17.5 20.9 versus 181.4 /H11006 17.5
Figure 7. Intracellular staining for specific cytokines of CD11c+
and CD4+ splenocytes stimulated with lipopolysaccharide. Splenocytes from DC-hBcl-2 Apoe−/− (n = 16) and Apoe+/− (n = 15) mice fed a Western diet for 4 to 9 weeks were stimulated for 15 hours with lipopolysaccharide. A–E, Flow cytometric analysis of gated CD4+ (A, C, E) and CD11c+ (B, D) mouse splenocytes expressing IL-10 (A and B), IL-17 (C), IL-12p40 (D), and IFN-γ (E). *P < 0.05, **P < 0.01, ***P < 0.0001.
both Th1 and Th17 phenotypes were upregulated in our model.
In conclusion, the main changes in immune system activation
on the Ldl-r−/− background (ie, enhanced Th1 activation along with Th1-driven IgG2c anti-oxidized LDL and anti-malondialdehyde-LDL production) were equally present on an Apoe−/− background. Additionally, we observed an increase in the activity of the Th17 pathway and a potential increase in Tr-1 regulatory T cells.

Effect of DC Lifespan and Immunogenicity on Atherosclerotic Lesion Progression, Plasma Lipids, and Lipoprotein Profile in Apoe−/− Mice
We analyzed lesion area in chow-fed 20-week-old DC-hBcl-2 Apoe−/− and Apoe+/− mice and reported the absence of a significant difference between the 2 groups of animals (Figure 8A; 149.5 ± 20.9 versus 181.4 ± 17.5 × 103 μm2, respectively; P = 0.3). Moreover, comparison of plaque burden in DC-hBcl-2 Apoe−/− and Apoe+/− mice fed a Western diet for 8 weeks revealed no difference in lipid deposition between groups (Figure 8B; 184.1 ± 21.7 versus 247.4 ± 35.3 × 103 μm2, respectively; P = 0.13). Taken together, and despite marked elevation in T-cell activation and a Th1-polarized immune response in DC-hBcl-2 mice compared with controls, no significant difference in lesion areas was found between groups. On the contrary, we observed a consistent trend toward attenuated lesion progression in DC-hBcl-2 mice compared with controls in all mouse models studied. We next compared plasma lipid levels in Apoe−/− and DC-hBcl-2

Figure 8. Quantification of atherosclerotic lesion surface, modulation of plasma cholesterol levels, and lipoprotein cholesterol profiles in DC-hBcl-2 Apoe−/− and Apoe+/− mice. The degree of atherosclerosis was determined by oil red O staining of aortic root sections from chow-fed 20-week-old DC-hBcl-2 Apoe−/− mice and Apoe+/− controls (A) and from DC-hBcl-2 Apoe−/− mice and Apoe+/− controls fed a Western diet for 8 weeks (B). Each symbol represents mean lesion area in a single mouse; horizontal bar indicates mean value for the respective group. C, Cholesterol distribution across VLDL, LDL, and HDL lipoprotein classes was analyzed by gel filtration in DC-hBcl-2 Apoe−/− and Apoe+/− mice fed a Western diet for 8 weeks. D, DC depletion was achieved by DT injection in CD11c-DTR Apoe−/− mice fed a Western diet, and plasma total cholesterol was determined and compared with Apoe−/− controls injected with DT. E, Time course of changes in plasma cholesterol levels in DT-treated Apoe−/− and DC-hBcl2 Apoe−/− mice maintained on a chow diet; values represent mean ± SEM of 3 to 6 mice per group. Statistically significant differences between CD11c-DTR Apoe−/− and control groups; *P < 0.01, **P < 0.001, ***P < 0.0001. Unpaired (A, B, and D) and paired (E) 2-tailed Student t tests were used.

Apoe−/− fed either a chow or a Western diet and observed that plasma total and free cholesterol levels were significantly decreased in DC-hBcl-2 Apoe−/− mice compared with controls in both conditions, whereas triglyceride levels were unchanged (Table). Analysis of cholesterol distribution among plasma lipoprotein classes in DC-hBcl-2 Apoe−/− animals fed the Western diet revealed a reduction in the cholesterol content of particles in the size range of both VLDL and LDL (−37% and −22%, respectively; Figure 8C). It is interesting to note that we did not observe such changes in plasma lipid levels in DC-Bcl-2 mice on a wild-type background maintained on a chow diet (online-only Data Supplement Figure VI).

To confirm whether depletion of DCs in the Apoe−/− background would also result in an increase in cholesterol
levels as seen in the LDL-r−-deficient background (Figure 5B), cholesterol-fed CD11c-DTR Apoe−/− mice and Apoe−/− controls were injected with DT. An increment of 63% in plasma cholesterol concentration was observed in DT-treated CD11c-DTR Apoe−/− mice compared with DT-treated Apoe−/− controls after 24 hours (1102±110 versus 676±64 mg/dL, respectively, P=0.01; Figure 8D). Next, we analyzed the time course of changes in plasma cholesterol levels in another set of CD11c-DTR Apoe−/− mice fed a normal chow and observed a transient increase (Figure 8E) that was statistically significant 24 and 48 hours after DT injection. This time course is consistent with published data on the duration of CD11c-positive cell depletion in DT-treated CD11c-DTR mice.21 Such depletion was specific to conventional DCs and did not affect plasmacytoid DCs (online-only Data Supplement Figure VII). Considered together, these results reveal that modulation of conventional DC number impacts plasma cholesterol levels under conditions of hypercholesterolemia.

Discussion

A central question in the pathogenesis of atherosclerosis concerns the potential impact of DCs. Although DCs are present in human and murine atherosclerotic lesions,5–7 a paucity of experimental data exists on the role of DCs in atherosclerotic plaque progression. Therefore, we designed genetically modified mouse models to address this question. For this purpose, we examined the relationship between DC lifespan and progression of atherosclerosis and made several novel findings: (1) The extended lifespan of DCs impacts on T-cell activation status and polarization of the immune response toward the Th1 pathway; (2) DCs markedly alter the degree of cholesterolemia in mice; and (3) the hypocholesterolemic action of DCs compensates for the proatherogenic degree of cholesterolemia in mice; and (3) the hypocholesterolemic action of DCs compensates for the proatherogenic bias.22 This recent evidence suggests that they are associated with protection against atherosclerosis.18,26 In an atherosclerotic context, we found no significant changes in the percentage of the splenic natural Treg population (CD4+ Foxp3+ CD25+) in DC-hBcl-2 mice, consistent with previous studies showing that DC lifespan and immunogenicity did not alter the natural Treg compartment.9,10 Nevertheless, expression of IL-10 mRNA was upregulated in the spleen of DC-hBcl-2→Ldl-r−/− mice compared with control mice, and IL-10–producing CD4+ T cells were increased in the spleen of DC-hBcl-2 Apoe−/− mice. Such enhancement would predict protection against lesion development, as suggested by studies in which the IL-10 axis was modulated.21 Moreover, expansion of CD4+ IL-10+ T cells, also termed Treg cells or adaptive Tregs, could exert an antiatherogenic effect in our model. Indeed, this specific T-cell compartment has been described as a potent antiatherogenic population that may help to combat Th1 proatherogenic bias.27

Th2 or Th1 responses are associated with an immunoglobulin class switching to IgG1 or IgG2c, respectively.28,29 We therefore quantified titers of serum antibodies directed against oxidation-specific epitopes. Statistically significant increases occurred in titers of anti-malondialdehyde-LDL IgG2c and anti-oxidized LDL IgG2c in DC-hBcl-2 mice on Ldl-r− or Apoe-deficient backgrounds, which corroborates the development of a Th1 bias immune response in these mice observed at the level of cytokine expression (IFN-γ, IL-12, IL-15, and TIM-3).

As a major result of the present study, we unexpectedly observed that elevation in the DC population led to markedly decreased plasma cholesterol levels in both the Ldl-r−/− and Apoe−/− backgrounds. Using a mouse model that allowed a reverse approach (ie, specific depletion of DCs), we observed that conventional DC elimination induced elevation in plasma cholesterol levels, thereby arguing that conventional DCs may contribute to correction of hyperlpidemia and that such cells may be implicated in cholesterol homeostasis. Interestingly, the impact of expansion of the DC population on plasma cholesterol levels is consistent with other observations in both mice and humans supporting a role for mononuclear phagocytes (macrophages, DCs, and Kupffer cells) in cholesterol homeostasis. For example, granulocyte-macrophage colony-stimulating factor, a key factor for DC growth.
and differentiation, has been reported to exhibit a cholesterol-lowering effect in patients with aplastic anemia, a finding later confirmed in rabbits and in patients with coronary artery disease. Similarly, the hematopoietic growth factor macrophage colony-stimulating factor was also reported to lower cholesterol levels in rabbits and nonhuman primate models. In mice, the opposite effect was observed in the op/op strain mutated for macrophage colony-stimulating factor, in which monocytes and tissue macrophage populations such as Kupffer cells are reduced. Indeed, when bred on an Apoe−/− background, the op/op Apoe−/− mice present a 3-fold increase in cholesterol levels. Considered together, these data indicate a strong relationship between the mononuclear phagocyte system and the potential control of cholesterol homeostasis. In the present study, we report for the first time the implication of conventional DCs as a cell type able to favor cholesterol lowering in a hyperlipidemic environment. The precise mechanisms that underlie the decrement in plasma cholesterol levels in our mouse model were not explored in the framework of the present study; however, because they are present in many tissues (spleen, liver, gut, and intestine), DCs might favor lipoprotein uptake and clearance from the circulation. Of note, Stoneman et al reported no significant change in cholesterol levels in CD11b-DTR Apoe−/− mice treated with DT. In this latter model, DT-induced CD11b+ cell depletion was restricted to monocyte/macrophages, neutrophils, and CD11b+ conventional DCs, thereby indicating that depletion of these myeloid cells does not reproduce the effect observed on cholesterol levels in the CD11c-DTR Apoe−/− mice in the present study.

In addition to the role of DCs in facilitating a Th1-polarized immune response, the prevailing paradigm that underlies the pathogenic effects of T cells, the present study revealed that the size of the conventional DC population was closely associated with regulation of cholesterol homeostasis. Overall, these antagonistic responses balanced each other out, with a null effect on atherosclerotic plaque progression. Clearly then, our findings identify the DC as a key player in atherosclerosis through its impact on both immune response regulation and cholesterol homeostasis.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

In healthy and pathological tissues, dendritic cells (DCs) are the most effective cells to present antigens and to initiate immune responses. DCs have an elevated capacity to stimulate T lymphocytes, natural killer lymphocytes, and B lymphocytes. Thus, they represent a potential tool for vaccination or immunotherapy in infectious disease, cancers, transplant rejection, autoimmune diseases, and immunoinflammatory diseases. During atherogenesis, immunoinflammatory mechanisms contribute to the progression of atherosclerotic lesions; however, the precise role of DCs in the progression of atherosclerosis and related cardiovascular disease is indeterminate. To address this question, we created transgenic mice in which the lifespan of DCs was increased in response to elevated resistance to apoptosis (CD11c-hBcl2) on the one hand, and on the other, we used mice in which targeted depletion of DCs (CD11c-DTR [diphtheria toxin receptor]) could be achieved. The present data provide the first in vivo evidence that DCs profoundly and broadly impact immune responses in atherosclerosis and, unexpectedly, circulating cholesterol levels, a major cardiovascular risk factor. The impact of DCs on cholesterolemia level is relevant to data published in preclinical and clinical studies using granulocyte-macrophage colony stimulating factor, a well-known DC growth factor. Indeed, granulocyte-macrophage colony stimulating factor induced a reduction in circulating cholesterol levels in treated patients. Considered together, the potential role of DCs as a central regulator of both immunity and cholesterol homeostasis opens new therapeutic horizons in the treatment of atherosclerosis.
Conventional Dendritic Cells at the Crossroads Between Immunity and Cholesterol Homeostasis in Atherosclerosis

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SUPPLEMENTAL MATERIAL

Conventional Dendritic Cells at the Crossroads between Immunity and Cholesterol Homeostasis in Atherosclerosis.

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SUPPLEMENTAL METHODS

Transgenic DC-hBcl-2 mice

The hBcl-2 cDNA from the pORF-hBcl-2 plasmid (Invivogen) was cloned downstream of the mouse dendritic cell-specific promoter CD11c \(^1,2\) (up to -821 bp from the ATG codon) amplified from a mouse C57BL/6 BAC clone (BAC PAC Resources). The linearized transgenic construct was injected into C57BL/6 fertilized female pronuclei, and progeny positive for the transgene was crossed with C57BL/6 mice \(^3\).

Animals and bone marrow transplantation

For bone marrow transplantation, Ldl-\(r^{-/-}\) mice on the C57BL/6 background were supplied from Jackson Laboratories and bred in house. Female Ldl-\(r^{-/-}\) mice (8-9-week-old) were subjected to medullar aplasia with 10 Gray lethal total body irradiation as previously described \(^3\). The next day, femurs were isolated from donor DC-hBcl-2 or wt mice and 2.5 x 10\(^6\) bone marrow cells were injected intravenously into irradiated mice (100% C57BL/6) to rescue their haematopoietic systems. These mice were then housed in cages under air-filtered
conditions for 4 weeks in order to allow reconstitution of their haematopoietic system, after
which they were fed a Western diet consisting of 0.15% cholesterol and 20% saturated fat for
12 weeks. Equally, DC-hBcl-2 mice were crossed with Apoe−/− mice on the C57BL/6
background supplied from Charles River. DC-hBcl-2 Apoe−/− mice and Apoe−/− littermates were
fed either a chow diet to 20 weeks of age, or 6 week-old animals were switched to a Western
diet consisting of 0.15% cholesterol and 20% saturated fat for 8 weeks. To deplete the DC
population in vivo, we crossbred CD11c-DTR transgenic mice (100% C57BL/6) 4 with Apoe−/−
mice. Mice were injected intraperitoneally with either PBS or diphteria toxin (DT, 4ng/g) as
previously described 4. All animal procedures were performed with accreditation from the
French Government and under strict compliance with Animal Welfare Regulations.

Assessment of chimerism.
Genomic DNA from bone marrow cells was isolated using the Nucleospin DNA Kit
(Macherey Nagel). Chimerism was evaluated by quantification of the amount of Ldl-r ko
DNA in both wt→Ldl-r−/− and DC-hBcl-2→Ldl-r−/− mice by polymerase chain reaction. PCR
was performed using 100 ng of DNA per reaction. The specific primers used were as follows:
Ldl-r ko forward primer 5'-CCATATGCATCCCCAGTCTT-3' and Ldl-r ko reverse primer
5'-AATCCATCTTGTATCTCGGATC-3'. Amplification products obtained were
compared to define a ratio of DNA from wt mice diluted in DNA from Ldl-r−/− mice ranging
from 0 to 50% in order to determine the level of chimerism.

Plasma lipid analyses and determination of lipoprotein profiles.
Blood samples were collected following an overnight fast. Plasma total cholesterol, free
cholesterol and triglyceride concentrations were measured by enzymatic colorimetric assays
as previously described 5. Plasma lipoproteins were fractioned by gel filtration on two
Superose 6 columns (Amersham Biosciences) connected in series using a BioLogic DuoFlow Chromatography System (BioRad)\textsuperscript{5}.

**Analysis of atherosclerotic plaques and immunohistochemistry.**

Mice were sacrificed under isoflurane anesthesia and perfused with sterile ice-cold PBS. Hearts were collected, fixed in 10% formalin solution for 30 minutes followed by overnight incubation in a PBS/20% sucrose solution at 4°C and then embedded in Tissue-Tek OCT compound. Atherosclerotic lesions were quantified through the aortic root using Oil Red O staining as previously described\textsuperscript{6}. Briefly, approximately 60 sections, 10 µm thick, were cut through the proximal aorta. Every tenth section was stained with Oil Red O for 4 hours and then counterstained with Mayer haematoxylin for 1 minute. The extent of atherosclerosis was measured with color thresholding to delimit areas of ORO staining. For immunohistochemistry, aortic root cryosections were air-dried and fixed in 10% formalin for 30 minutes. Sections were then blocked for 60 minutes with 3% BSA in PBS and then incubated with anti-CD68 antibody (Serotec, 1:300) or control antibody overnight at 4°C. After washing, a biotinylated goat anti-rat Ig secondary antibody was added and the signal was enhanced using the tyramide signal amplification kit (Perkin Elmer) according to the manufacturer's protocol; sections were subsequently counterstained for nuclei with DAPI.

**Flow cytometry analysis.**

At sacrifice, splenocytes were harvested, minced and filtered through a 70-µm cell strainer. After red cell lysis in hypotonic buffer, cells were resuspended in PBS/1% BSA, pre-incubated for 5 min with Fc blocker and then incubated for 30 min at 4°C with antibodies directed against either CD45 (clone 30F11, Miltenyi), CD3 (clone 145-2C11, BD Pharmingen), CD4 (clone GK1.5, Miltenyi), CD11c (clone N418, eBiosciences), CD19 (clone
6D5, eBiosciences), Gr-1 (clone RB6-8C5, eBiosciences), PDCA-1 (clone JF05-1C2.4.1, Miltenyi); CD69 (clone H1.2F3, eBiosciences), CD44 (clone IM7, eBiosciences), CD25 (clone 7D4, Beckman Coulter), CD86 (clone GL1, eBiosciences), IL10 (clone JES5-16E3, eBiosciences), IL12p40 (clone C17,8, eBiosciences), IFNγ (clone XMG-1, eBiosciences), IL17 (clone TC11-18H101, BD Pharmingen), or Foxp-3 (clone FJK-16s, eBiosciences) in order to analyse the proportions of diverse splenocyte subpopulations. Cells were analysed on a FC 500 flow cytometer (Beckman Coulter) using CXP software. For single cell staining, splenocytes were isolated and cultured for 5 h or 15 hours in complete medium containing LPS (1µg/ml) and Brefeldin A. Intracellular staining was performed with a fixation and permeabilization kit (eBioscience) according to the manufacturer's instructions.

**Analysis of gene expression by quantitative real time PCR (Q-PCR).**

RNAs were prepared using TRIzol reagent (Invitrogen) from frozen tissue specimens isolated from mice at sacrifice. Each RNA preparation was hybridized with random hexamer (Promega) and reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). Quantitative real time PCR was performed using a LightCycler PCR System (Roche) as previously described. Hprt (hypoxanthine guanine phosphoribosyl transferase) was used as a housekeeping gene in order to account for variability in the initial quantities of cDNA. Expression data were based on the crossing points calculated from the LightCycler data analysis software and corrected for PCR efficiencies of both the target and the reference gene.

**Quantification of anti-MDA LDL, anti-oxLDL and EO6 antibodies.**

Oxidatively modified-LDL-specific antibodies were determined as described. Wells were coated with either malondialdehyde-LDL (MDA-LDL) or copper-oxidized-LDL (oxLDL) at 5µg/ml in PBS-EDTA. Sera were diluted 1:100 and binding of IgM, IgG1, IgG2c and IgG3 to
wells coated with antigens was detected by chemiluminescent enzyme immunoassays using alkaline phosphatase (AP)-labeled secondary antibodies and the substrate LumiPhos (Lumigen). For the measurement of serum T15 clonotypic (EO6) antibodies, a double antibody capture assay was used. Wells were coated with 2 µg/ml monoclonal anti-T15-idiotypic antibody AB1-2 (ATCC). Sera were diluted 1:100 and binding of T15 clonotypic IgM antibodies was detected by using 0.1µg/ml biotinylated AB1-2 followed by alkaline phosphatase-labeled NeutrAvidin and the substrate LumiPhos. For all measurements, luminescence was determined using a Dynatech luminometer (Dynex Technologies) and antibody binding was measured as relative light units (RLUs) measured over 100 ms.

**Generation of bone marrow-derived dendritic cells (BMDCs)**

BMDCs were generated as described by previously 9. Briefly, at day 0, bone marrow cells were seeded at 2 x 10^6 per 100 mm dish in 10 ml complete R10 medium (RPMI 1640 supplemented with Penicillin -100 U/ml-, Streptomycin -100 µg/ml-, L-glutamine -2 mM-, 2-mercaptoethanol -50 µM-, 10% heat-inactivated FCS and 20 ng/ml mGM-CSF). At day 3, another 10 ml R10 medium were added to the plates. At days 6 and 8, half of the culture supernatant volume was collected and centrifuged. Subsequently, the cell pellet was resuspended in 10 ml fresh R10 medium and transferred back to the original plate. Cells were used at day 10.

**Statistical analysis.**

The statistical significance of the differences between groups was evaluated using the two-tailed Student t-test; P<0.05 was considered significant. Values are expressed as mean ± SEM.
Supplementary Figure I. hBcl-2 expression in different cell types of the immune system. The shaded profile represents wt mice and the open profile DC-hBcl-2 mice. Intracellular staining of hBcl-2 was performed in splenic dendritic cells isolated using anti-CD11c antibody-coated magnetic beads (A). Other specific leucocyte (CD45+) populations in spleen, peritoneal lavage, lung and blood were identified by combinations of cell-specific antibodies (B).
Supplementary Figure II. Blood leucocyte and monocyte counts in DC Apoe<sup>−/−</sup> mice fed a Western diet. Circulating leucocyte (A), monocyte (CD115<sup>+</sup>) (B, Ly6C<sup>hi</sup> (Gr1<sup>hi</sup>) monocytes (C), and Ly6C<sup>lo</sup> (Gr1<sup>lo</sup>) monocyte (D) numbers.
Supplementary Figure III. Intracellular staining of hBcl-2 in bone marrow derived dendritic cells from wt and DC-hBcl-2 mice (A). The shaded profile represents wt mice and the open profile DC-hBcl-2 mice. BMDCs derived from wt and DC-hBcl-2 mice were submitted to serum and growth factor deprivation for 24 and 48 hours and survival was determined by Annexin-V/PI staining (B). Controls are BMDCs derived from wt grown in complete medium containing serum and growth factors. DC-hBcl-2 mice exhibited greater DC abundance (C) and enhanced T cell activation at steady state (D). Statistically significant differences: * P<0.05, ** P<0.01 and *** P<0.001.
**Supplementary Figure IV.** Assessment of chimerism in DC-\(hBcl-2\rightarrow Ldl-r^-\) mice. The amount of \(Ldl-r\) ko DNA was determined in DNA from bone marrow cells of \(wt\rightarrow Ldl-r^-\) and \(DC-hBcl-2\rightarrow Ldl-r^-\) mice. More than 95% of the \(Ldl-r\) alleles in \(DC-hBcl-2\rightarrow Ldl-r^-\) mice were of donor origin.
Supplementary Figure V. Levels of mRNA expression of functional markers for dendritic cells (A) and regulatory T cells (B) were evaluated by qPCR in spleens from DC-\textit{hBcl-2} Apoe\textsuperscript{-/-} (n=7) and Apoe\textsuperscript{-/-} (n= 10) mice fed a Western diet for 4 to 9 weeks. Statistically significant differences: * \(P<0.05\).
Supplementary Figure VI. Circulating levels of total cholesterol, free cholesterol and triglyceride in chow-fed, 6 week old, DC-\textit{hBcl}-2 mice as compared to littermate controls.
Supplementary Figure VII. Dendritic cells populations (cDCs and pDCs) in the liver and spleen of CD11c-DTR mice 24 hours after i.v. injection of DT or vehicle. Flow cytometric analysis revealed that pDCs are resistant to DT-induced depletion while cDCs are depleted in both spleen and liver.
SUPPLEMENTAL REFERENCES