B-Cell Aortic Homing and Atheroprotection Depend on Id3

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Rationale: B cells are abundant in the adventitia of normal and diseased vessels. Yet, the molecular and cellular mechanisms mediating homing of B cells to the vessel wall and B-cell effects on atherosclerosis are poorly understood. Inhibitor of differentiation-3 (Id3) is important for atheroprotection in mice and polymorphism in the human ID3 gene has been implicated as a potential risk marker of atherosclerosis in humans. Yet, the role of Id3 in B-cell regulation of atherosclerosis is unknown.

Objective: To determine if Id3 regulates B-cell homing to the aorta and atheroprotection and identify molecular and cellular mechanisms mediating this effect.

Methods and Results: Loss of Id3 in Apoe−/− mice resulted in early and increased atherosclerosis. Flow cytometry revealed a defect in Id3−/− Apoe−/− mice in the number of B cells in the aorta but not the spleen, lymph nodes, and circulation. Similarly, B cells transferred from Id3−/− Apoe−/− mice into B-cell–deficient mice reconstituted spleen, lymph node, and blood similarly to B cells from Id3+/+ Apoe−/− mice, but aortic reconstitution and B-cell–mediated inhibition of diet-induced atherosclerosis was significantly impaired. In addition to retarding initiation of atherosclerosis, B cells homed to regions of existing atherosclerosis, reduced macrophage content in plaque, and attenuated progression of disease. The chemokine receptor CCR6 was identified as an important Id3 target mediating aortic homing and atheroprotection.

Conclusions: Together, these results are the first to identify the Id3-CCR6 pathway in B cells and demonstrate its role in aortic B-cell homing and B-cell–mediated protection from early atherosclerosis. (Circ Res. 2011;109:00-00.)

Key Words: atherosclerosis ■ B lymphocytes ■ transcription factors ■ helix-loop-helix ■ homing

Atherosclerosis is a progressive, chronic inflammatory disease resulting in plaque formation in large arteries1–2 that can lead to heart attack, stroke, and death. Targeting the immune cells that participate in atherogenesis is a promising novel approach for atherosclerosis treatment and prevention.3 Much is known about the recruitment of macrophages and T cells to the vessel wall and their role in plaque progression;4 however, B cells in atherosclerosis are incompletely understood. As early as the 1960s, studies have identified B cells, plasma cells, and immunoglobulins in association with atherosclerotic plaques of mice and humans.5–14 More recent studies in mice identified B-cell–containing aortic tertiary lymphoid organs (ATLO) in the adventitia adjacent to atherosclerotic plaques, raising the possibility that adventitial B cells may regulate a local immune response within the vessel wall.5,6,14 In addition, although ATLOs are not present in the normal/noninflamed aorta before Western feeding,5 a significant population of leukocytes, including B cells, are present in the aortic adventitia of normal vessels.14 Global B-cell deficiency established before Western diet feeding15,16 results in increased atherosclerosis in mice, suggesting that B cells function in early atheroprotection. Yet, the molecular mechanisms that regulate homeostatic trafficking of B cells to the aorta and their impact on the development of atherosclerosis are unknown.

Inhibitor of differentiation-3 (Id3), a member of the helix-loop-helix (HLH) family of transcription factors, is a domi-
nant negative inhibitor of bHLH protein-DNA binding and gene expression in B cells.17 The Id3−/− mouse has normal numbers and maturity of B cells but develops a Sjögren-like syndrome with lachrymal and salivary glands lymphocytic infiltrates,13 raising the interesting possibility that Id3 may regulate B-cell homing to sites of disease. That hyperlipemia increased Id3 expression in vitro and in the vessel wall in a porcine model suggested a link between Id3 and atherosclerosis.20 This link was recently confirmed by studies demonstrating that aged and Western diet–fed Apoe−/− mice null for Id3 had significantly increased atherosclerosis compared with Apoe−/− mice wild-type for Id3.21 Moreover, Id3 may be involved in atheroprotection in humans as the human ID3 gene contains a single nucleotide polymorphism (SNP) that alters Id3 protein function and is associated with increased carotid intima-media thickness in humans.22

The present study demonstrates a new function for Id3, as a critical regulator of B-cell aortic trafficking and B-cell–mediated atheroprotection and identifies CCR6 as an Id3 target gene mediating these effects. In addition, we provide the first evidence that B cells home to regions prone to and with existing atherosclerosis leading to reduced macrophage accumulation and attenuation of lesion progression.

Methods
Detailed experimental procedures and associated references are in the Online Supplemental Material available at http://circres.ahajournals.org. All procedures using animals were carried out according to protocols approved by the Animal Care and Use Committee at the University of Virginia. For B-cell adoptive transfer studies, spleens were harvested from 10- to 12-week-old mice and B cells were isolated by negative selection using MACS anti-CD43 microbeads or a combination of MACS anti-CD43, anti-CD4, and anti-CD11b microbeads (Miltenyi Biotec). Serum cholesterol levels were determined using an Archetect 8000 series analyzer. Antibody titers were determined as detailed in the Online Supplemental Material.

Analysis of Atherosclerosis
After euthanizing the mice, the aorta was harvested from the heart to the iliac bifurcation. Both en face Sudan IV staining of the aorta and cross-sectional analysis of the root was used in this study to quantify atherosclerosis. Fluorescent imaging at the UVA Advanced Microscopy Core enabled identification of CFDA-SE-labeled B cells within the aorta. Immunohistochemical analysis of atherosclerosis was achieved through staining for MCP-1 (Santa Cruz Biotec) and macrophage content was determined with Mac-2 staining (Cedarlane Laboratories). These protocols are detailed in the Online Supplemental Material.

Bone Marrow Transplantation
Id3+/- Apoe−/− and Id3−/− Apoe−/− mice were subjected to a sublethal dose of radiation (500 rads × 2 irradiations) and subsequently reconstituted with 5 × 10^6 bone marrow cells harvested from the femurs and tibias of Id3+/- Apoe−/− and Id3−/− Apoe−/− donor mice, as detailed in the Online Supplemental Material.

Flow Cytometry
Lymph nodes, spleens, blood, and aortas, including the adventitia, were harvested under a dissection microscope and processed for flow cytometry as previously described14 and detailed in the Online Supplemental Material.

Ex Vivo Imaging of Radiolabeled B Cells
B cells were radiolabeled by incubation in indium-111 oxine solution. After adoptive transfer of radiolabeled B cells, aortas were harvested and exposed to a high-sensitivity, medium-resolution phosphor imaging screen (PerkinElmer) overnight. The phosphor imaging screen was scanned using a PerkinElmer Cyclone Plus Phosphor Imaging System. This protocol is detailed in the Online Supplemental Material.

Real-Time PCR
Total cellular RNA was collected from B cells using an RNaseasy kit (Qiagen) as per the manufacturer’s instructions, cDNA was then synthesized using an iScript cDNA synthesis kit (BioRad), and real-time PCR reaction using a Bio-Rad iCycler and iQ SYBR Green Supermix (BioRad) was performed as detailed in the Online Supplemental Material.

Cellular Migration Studies
Splenic B cells were purified from Id3+/+ Apoe−/− and Id3−/− Apoe−/− mice and placed in the upper chamber of 5-μm pore size transwells containing either 1000 ng/mL of CXCL13 or 500 ng/mL of CCL20 in the bottom of the transwell. After incubating for 6 hours, the number of cells that had migrated through the transwell was counted, using a flow cytometer as detailed in the Online Supplemental Material.

Statistical Analysis
A probability value < 0.05 was considered statistically significant. All statistical analyses were performed using NCSS 2001 (Number Crunching Statistical Software, Kaysville, UT) and GraphPad Prism 5 (La Jolla, CA). Statistical analysis is detailed in the Online Supplemental Material.

Results
Id3 Is Necessary for Early Atheroprotection
To determine if Id3−/− Apoe−/− mice developed premature atherosclerosis, Id3+/- Apoe−/− and Id3−/− Apoe−/− mice were fed a Western diet for 4 or 8 weeks, then harvested for en face analysis of lesion area (Figure 1A). After 4 weeks of Western feeding, Id3−/− Apoe−/− but not Id3+/+ Apoe−/− mice developed a discernable amount of lesion (0.053 ± 0.009% versus 1.16 ± 0.483%: P = 0.004). After 8 weeks of Western feeding, both genotypes developed detectable atherosclerosis, with significantly more lesion seen in the Id3−/− Apoe−/− mouse (0.347 ± 0.111% versus 4.03 ± 0.859%; P = 0.002) (Figure 1B). There were no differ-
Id3 Atheroprotection Is Predominantly Mediated by a Bone Marrow–Derived Cell

Bone marrow chimeras fed a Western diet for 16 weeks confirmed that Id3 deletion increased atherosclerosis and revealed that Id3−/− Apoe−/− mice reconstituted with bone marrow from Id3+/+ Apoe−/− mice had a significant attenuation of atherosclerosis compared with those reconstituted with Id3−/− Apoe−/− marrow (3.1-fold reduction). Similarly, Id3+/+ Apoe−/− mice developed significantly less atherosclerosis when reconstituted with bone marrow from Id3+/+ Apoe−/− compared with Id3−/− Apoe−/− mice (5.2-fold reduction) (Figure 2E). There was a significant increase in atherosclerosis when Id3+/− Apoe−/− compared with control Id3+/+ Apoe−/− recipient mice were reconstituted with bone marrow from Id3+/+ Apoe−/− mice, suggesting some contribution of loss of Id3 in cells not derived from bone marrow, although this effect was much less marked than when the bone marrow cells lacked Id3. No differences in weight, lipid parameters, glucose or insulin were noted in the bone marrow chimera (Online Table II).

Id3−/− Apoe−/− Mice Have Significantly Fewer B Cells in the Aorta Compared With Id3+/+ Apoe−/− Mice

The early onset atherosclerosis in the Id3−/− Apoe−/− mice raised the interesting hypothesis that Id3 may regulate immune cells resident in the aorta before lesion development. Flow cytometry of aortas from chow-fed Id3−/− Apoe−/− and Id3+/+ Apoe−/− mice demonstrated equivalent numbers of total leukocytes (CD45+) (Figure 2A), T cells (Figure 2B), and dendritic cells (Figure 2C). The number of macrophages was higher in the aortas of Id3−/− Apoe−/− mice (Figure 2D). In contrast, the number of B cells in the aorta was significantly lower in Id3−/− Apoe−/− mice (Figure 2E and 2F). This finding cannot be explained by a global reduction in the number of B cells in the Id3−/− Apoe−/− mouse, as consistent with previous findings in Id3−/− mice,18 the number of B cells in the spleen, peri-aortic lymph nodes, and whole blood...
were similar between \( \text{id}^{3+/-}\) \text{Apo}^{e/-} and \( \text{id}^{3-/-}\) \text{Apo}^{e/-} mice (Table 1).

Optical Imaging Confirms Fewer B Cells in Aorta of \( \text{id}^{3-/-}\) \text{Apo}^{e/-} Mice and Reveals That the B Cells Present Predominantly Localize to Atheroprone Regions of the Aorta

Optical imaging using a Cy5.5-labeled anti-B220 antibody provided a second method to demonstrate B cells in the aorta of young chow-fed \( \text{id}^{3-/-}\) \text{Apo}^{e/-} and \( \text{id}^{3+/-}\) \text{Apo}^{e/-} mice. As a control, \( \mu\text{MT}\) mice, which lack peripheral B cells due to deletion of genomic DNA sequences that encode the transmembrane domain of the B-cell receptor \( \mu\) heavy chain,\(^22\) were bred to \text{Apo}^{e/-} mice to generate B-cell-deficient \( \text{Apo}^{e/-}\) mice (\( \mu\text{MT Apo}^{e/-}\)). B-cell-deficient \( \mu\text{MT Apo}^{e/-}\) aortas were found to have a minimal amount of background signal, which probably represents the nonspecific retention of the Cy5.5-labeled anti-B220 antibody. Although a significant increase in fluorescence over background was observed in both \( \text{id}^{3+/-}\) \text{Apo}^{e/-} and \( \text{id}^{3-/-}\) \text{Apo}^{e/-} aortas (Figure 2G), consistent with our previous flow cytometric data, the increase in fluorescence over controls in the aortas of \( \text{id}^{3-/-}\) \text{Apo}^{e/-} mice was significantly lower than that in the aortas of \( \text{id}^{3+/-}\) \text{Apo}^{e/-} mice (15% versus 42%; \( P<0.0006\)) (Figure 2G and 2H). Notably, peak fluorescence was greatest within the aortic arch and the abdominal aorta (Figure 2G), regions that are particularly prone to the development of atherosclerosis in this animal model.\(^23\)

### Table 1. Number of B Cells in the Spleen, Peri-Aortic Lymph Nodes, and Whole Blood

<table>
<thead>
<tr>
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<th>( \text{id}^{3+/-}) \text{Apo}^{e/-}</th>
<th>( \text{id}^{3-/-}) \text{Apo}^{e/-}</th>
<th>P Value</th>
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<td>Peripheral B lymphocytes, ( \times 10^{6} ) CD19 \text{+} CD45 \text{+} cells</td>
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<tr>
<td>Spleen</td>
<td>56.5±10.4</td>
<td>56.3±9.31</td>
<td>NS</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>0.89±0.11</td>
<td>0.88±0.14</td>
<td>NS</td>
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<tr>
<td>Blood, per mL</td>
<td>1.96±0.21</td>
<td>1.91±0.29</td>
<td>NS</td>
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Values are mean±SD. NS indicates not significant.
Id3 Is Necessary for B-Cell Homing to the Aorta

To determine if Id3 regulated homocystein transmigration of B cells to the aorta, adoptive transfer studies were conducted. Splenic B cells from Id3<sup>+/+</sup> ApoE<sup>−/−</sup> or Id3<sup>−/−</sup> ApoE<sup>−/−</sup> mice were adoptively transferred to μMT ApoE<sup>−/−</sup> mice. Control animals received an equal volume of vehicle. Six, 24, or 72 hours after the transfer of purified B cells, the aorta, spleen, lymph nodes, and whole blood were harvested (Figure 3A) and analyzed by flow cytometry as described above. ApoE<sup>−/−</sup> B cells wild-type for Id3 appear in the aorta 6 hours after tail vein injection and continue to accumulate in the aorta up to 72 hours later. In contrast, no appreciable numbers of Id3<sup>−/−</sup> ApoE<sup>−/−</sup> B cells appear in the aorta at any of the time points tested (Figure 3B). Analysis of the spleens, lymph nodes, and whole blood of the same animals revealed that B-cell reconstitution of these compartments was similar between the two genotypes at each time point, providing evidence that reduced B-cell number was not due to a reduction in the total B-cell pool (Table 2).

To confirm Id3-dependent trafficking of B cells to the aorta, imaging of radiolabeled B cells injected into μMT ApoE<sup>−/−</sup> mice was performed. Splenic B cells were isolated from Id3<sup>+/+</sup> ApoE<sup>−/−</sup> and Id3<sup>−/−</sup> ApoE<sup>−/−</sup> mice and radio-labeled with indium-111 (In-111) oxine. Recipient μMT ApoE<sup>−/−</sup> mice were injected with 1×10<sup>7</sup> In-111 radiolabeled B cells or with In-111 oxine in normal saline as control. Aortas were harvested 20 hours later, opened en face, and phosphor imaging was performed. Significantly more radioactive signal was present in the aortas of mice receiving Id3<sup>+/+</sup> ApoE<sup>−/−</sup> compared with Id3<sup>−/−</sup> ApoE<sup>−/−</sup> B cells (Figure 3C and 3D). Consistent with optical imaging data (Figure 2G), radiolabeled B cells traffic to regions of the aorta that are prone to atherosclerosis (Figure 3C and 3E). To determine the layer of the vessel wall to which B cells traffic in these experiments, splenic B cells were purified from Id3<sup>+/+</sup> ApoE<sup>−/−</sup> mice, incubated with CFDA-SE, and adoptively transferred to μMT ApoE<sup>−/−</sup> mice. Seventy-two hours after transfer, aortas were harvested from mice, sectioned and analyzed for the presence of these labeled B cells. Although the media and scattered adventitial cells revealed autofluorescence, CFDA-SE labeled B cells were only detected within the adventitia (Figure 3F). B cells in the spleen served as a positive control and spleen from mice receiving vehicle injection served as negative control.
Peripheral B lymphocytes, through tail vein injection to B-cell–deficient Id3-deficient mice,15,16 μMT Apoe−/− mice fed 16 weeks of a Western diet developed significant atherosclerosis. A single injection of 30×10^6 Id3+/+ Apoe−/− B cells to μMT Apoe−/− mice significantly reduced atherosclerotic lesion area (9.69±0.568% versus 5.10±0.836%; P<0.002). B-cell inhibition of atherosclerosis was dose-dependent, as there was an even greater reduction in atherosclerosis with the delivery of 60×10^6 Id3+/+ Apoe−/− B cells (9.69±0.568% versus 2.74±0.263%; P<0.001). In contrast, lesion area was not significantly changed in animals receiving either 30 or 60×10^6 Id3−/− Apoe−/− B cells (9.69±0.568% versus 8.18±0.735% or 7.82±1.16%) (Figure 4B and 4C). Flow cytometry confirmed a dose dependent increase in aortic B-cell number with increasing numbers of Id3+/+ Apoe−/− B cells injected. Injection of 30×10^6 Id3+/+ Apoe−/− B cells resulted in an average of 2034 aortic B cells (data not shown) and 60×10^6 Id3+/+ Apoe−/− B cells resulted in >5000 aortic B cells (Figure 3B), measured 72 hours after tail vein injection. For B cells from Id3−/− Apoe−/− mice, there were <200 B cells in the aorta regardless of the number of B cells injected (data not shown and Figure 3B). There were no differences in the numbers of peripheral B cells (Table 3). These data demonstrate that B cell delivery before Western diet feeding protects μMT Apoe−/− mice from diet-induced atherosclerosis and that Id3 is essential for this B-cell–mediated attenuation of atherosclerosis.

CCR6 Is a Novel Id3 Target Regulating B-Cell Homing to the Aorta and Atheroprotection

To determine whether Id3 is essential for B-cell–mediated attenuation of atherosclerosis, adoptive transfer studies in μMT Apoe−/− mice were performed. Splenic B cells from Id3+/+ Apoe−/− or Id3−/− Apoe−/− mice were transferred through tail vein injection to B-cell–deficient μMT Apoe−/− mice. Control mice received an equal volume of PBS vehicle. All recipient mice were then fed a Western diet for 16 weeks, after which atherosclerosis was assessed by en face analysis (Figure 4A). Consistent with previous studies of B-cell–deficient mice,15,16 μMT Apoe−/− mice fed 16 weeks of a Western diet developed significant atherosclerosis. A single injection of 30×10^6 Id3+/+ Apoe−/− B cells to μMT Apoe−/− mice significantly reduced atherosclerotic lesion area (9.69±0.568% versus 5.10±0.836%; P<0.002). B-cell inhibition of atherosclerosis was dose-dependent, as there was an even greater reduction in atherosclerosis with the delivery of 60×10^6 Id3+/+ Apoe−/− B cells (9.69±0.568% versus 2.74±0.263%; P<0.001). In contrast, lesion area was not significantly changed in animals receiving either 30 or 60×10^6 Id3−/− Apoe−/− B cells (9.69±0.568% versus 8.18±0.735% or 7.82±1.16%) (Figure 4B and 4C). Flow cytometry confirmed a dose dependent increase in aortic B-cell number with increasing numbers of Id3+/+ Apoe−/− B cells injected. Injection of 30×10^6 Id3+/+ Apoe−/− B cells resulted in an average of 2034 aortic B cells (data not shown) and 60×10^6 Id3+/+ Apoe−/− B cells resulted in >5000 aortic B cells (Figure 3B), measured 72 hours after tail vein injection. For B cells from Id3−/− Apoe−/− mice, there were <200 B cells in the aorta regardless of the number of B cells injected (data not shown and Figure 3B). There were no differences in the numbers of peripheral B cells (Table 3). These data demonstrate that B cell delivery before Western diet feeding protects μMT Apoe−/− mice from diet-induced atherosclerosis and that Id3 is essential for this B-cell–mediated attenuation of atherosclerosis.

Reconstitution of μMT Apoe−/− Mice With Id3+/+ Apoe−/− But Not Id3−/− Apoe−/− B Cells Inhibited Western Diet–Induced Atherosclerosis

To determine whether Id3 is essential for B-cell–mediated attenuation of atherosclerosis, adoptive transfer studies in μMT Apoe−/− mice were performed. Splenic B cells from Id3+/+ Apoe−/− or Id3−/− Apoe−/− mice were transferred through tail vein injection to B-cell–deficient μMT Apoe−/− mice. Control mice received an equal volume of PBS vehicle. All recipient mice were then fed a Western diet for 16 weeks, after which atherosclerosis was assessed by en face analysis (Figure 4A). Consistent with previous studies of B-cell–deficient mice,15,16 μMT Apoe−/− mice fed 16 weeks of a Western diet developed significant atherosclerosis. A single injection of 30×10^6 Id3+/+ Apoe−/− B cells to μMT Apoe−/− mice significantly reduced atherosclerotic lesion area (9.69±0.568% versus 5.10±0.836%; P<0.002). B-cell inhibition of atherosclerosis was dose-dependent, as there was an even greater reduction in atherosclerosis with the delivery of 60×10^6 Id3+/+ Apoe−/− B cells (9.69±0.568% versus 2.74±0.263%; P<0.001). In contrast, lesion area was not significantly changed in animals receiving either 30 or 60×10^6 Id3−/− Apoe−/− B cells (9.69±0.568% versus 8.18±0.735% or 7.82±1.16%) (Figure 4B and 4C). Flow cytometry confirmed a dose dependent increase in aortic B-cell number with increasing numbers of Id3+/+ Apoe−/− B cells injected. Injection of 30×10^6 Id3+/+ Apoe−/− B cells resulted in an average of 2034 aortic B cells (data not shown) and 60×10^6 Id3+/+ Apoe−/− B cells resulted in >5000 aortic B cells (Figure 3B), measured 72 hours after tail vein injection. For B cells from Id3−/− Apoe−/− mice, there were <200 B cells in the aorta regardless of the number of B cells injected (data not shown and Figure 3B). There were no differences in the numbers of peripheral B cells (Table 3). These data demonstrate that B cell delivery before Western diet feeding protects μMT Apoe−/− mice from diet-induced atherosclerosis and that Id3 is essential for this B-cell–mediated attenuation of atherosclerosis.

Table 2. Number of B Cells in the Spleen, Peri-Aortic Lymph Nodes, and Whole Blood After Adoptive Transfer to μMT Apoe−/− Mice

| Vehicle | μMT Apoe−/− | μMT Apoe−/− | | P Value |
|---------|-------------|-------------| |         |
| Peripheral B lymphocytes, ×10^6 CD19+ CD45+ cells |
| 6 Hours after transfer |
| Spleen |
| 39.9±0.59 | 37.6±0.22 | NS |
| Lymph node |
| 1.37±0.64 | 1.02±0.41 | NS |
| Blood, per mL |
| 1.50±0.40 | 1.60±0.30 | NS |
| 24 Hours after transfer |
| Spleen |
| 0.006±0.001 | 72.1±1.42 | 76.7±1.37 | NS |
| Lymph node |
| 0.005±0.001 | 1.11±0.12 | 1.22±0.19 | NS |
| Blood, per mL |
| 0.006±0.001 | 1.20±0.30 | 1.00±0.20 | NS |
| 72 Hours after transfer |
| Spleen |
| 94.5±0.50 | 93.4±0.58 | NS |
| Lymph node |
| 1.01±0.10 | 1.12±0.08 | NS |
| Blood, per mL |
| 1.40±0.70 | 1.50±0.40 | NS |

Values are mean±SEM. NS indicates not significant.

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December 9, 2011

Figure 4. Id3+/+ Apoe−/− but not Id3−/− Apoe−/− B cells confer atheroprotection to B-cell–deficient μMT Apoe−/− mice. A, Schematic of the experiment. At 8 weeks of age, 30×10^6 or 60×10^6 splenic B cells from either Id3+/+ Apoe−/− or Id3−/− Apoe−/− donors were adoptively transferred into μMT Apoe−/− recipients by tail vein injection. Control animals received an equal volume of PBS vehicle. Animals were allowed to recover for 72 hours, then fed a Western diet for 16 weeks. Aortas were harvested and analyzed by en face as described in Figure 1. B, Representative photos of aortic Sudan IV staining. C, Quantification of atherosclerotic lesion size. Each point represents a single animal. **P<0.05, ***P<0.002, ****P<0.0001.
we performed a murine PCR chemokines and receptor array (SA Biosciences) using RNA from B cells derived from Id3+/+ or Id3−/− mice. Notably, the chemokine receptor, CCR6, implicated in cell homing to sites of disease was significantly reduced. Real-time PCR confirmed the array results, identifying a 3-fold reduction in CCR6 mRNA expression in Id3−/− B cells compared with Id3+/+ B cells (Figure 5A). No change was noted in the levels of CXCR5, CXCR6, and L-selectin, which have previously been demonstrated to play a role in T-cell homing and transcript regulatory effects. Transient cotransfection studies in BJAB cells (a human B-cell lymphoma line), demonstrated that E12 inhibits CCR6 promoter activation, an effect antagonized by cotransfection with an expression plasmid encoding Id3 (Figure 5D). Flow cytometry of aorta from C57BL/6 (B6) and CCR6−/− mice revealed fewer aortic B cells in CCR6−/− mice than those of age-matched littermate B6 control mice (Figure 5E and 5F). Adoptive transfer of CCR6−/− B cells to μMT Apoe−/− mice yielded significantly fewer B cells within the aortas of mice receiving B cells from CCR6−/− compared with B6 mice (Figure 5G and 5H). Moreover, adoptive transfer of 60×10^6 Ccr6−/− B cells to μMT Apoe−/− mice significantly reduced atherosclerotic lesion area in response to 16 weeks of Western diet feeding (16.1±0.992% versus 9.28±0.783%; P=0.0002). In contrast, lesion area was not significantly changed in animals receiving Ccr6+/− B cells (16.1±0.992% versus 13.4±0.823%; P=NS) (Figure 5I). These data identify CCR6 as an Id3 target involved in the aortic “address” for B-cell homing and B-cell-mediated atheroprotection.

### Table 3. Number of B Cells in the Spleen, Peri-Aortic Lymph Nodes, and Whole Blood After Adoptive Transfer to μMT Apoe−/− Mice and 16 Weeks of Western Diet Feeding

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<td>Peripheral B lymphocytes, ×10^6</td>
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<tr>
<td>CD19+ CD45+ cells</td>
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<td></td>
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<tr>
<td>30×10^6 B cells transferred</td>
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<tr>
<td>Spleen</td>
<td>11.1±1.31</td>
<td>10.6±1.23</td>
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<tr>
<td>Lymph nodes</td>
<td>1.86±0.12</td>
<td>1.75±0.17</td>
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<td>Blood, per mL</td>
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<td>0.20±0.10</td>
<td>NS</td>
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Values are mean±SEM. NS indicates not significant.

**B Cells Attenuate Aortic Macrophage Content**

The mechanisms whereby aortic B cells attenuate plaque development are unknown. As Id3−/− mice have increased total IgM in serum,^{25} we investigated the serum levels of antibodies known to be correlated with atherosclerosis in Id3+/+ and Id3−/− Apoe−/− chow-fed mice. Paradoxically, IgM MDA-LDL and IgM Cu-OxLDL, which are associated with lower levels of atherosclerosis,^{26} were found to be increased in Id3+/+ Apoe−/− mice compared with Id3−/− Apoe−/− mice. Levels of IgG MDA-LDL and IgG Cu-OxLDL were not significantly different between groups (Online Table I). Macrophages are present in the aorta before Western diet feeding^{14} (Figure 2D) and infiltrate the vessel...
wall early in the disease process. To determine if B-cell delivery to MT Apoe−/− mice alters plaque macrophage content, immunohistochemical staining of plaques from vehicle and B-cell injected MT Apoe−/− mice was performed. Compared with control, adoptive transfer of B cells resulted in a significant reduction in Mac2 (Figure 6F and 6G, 0.34±0.02 versus 0.42±0.02, respectively, P=0.024), and MCP-1 staining (Figure 6F and 6H, 0.074±0.009 versus 0.121±0.012, P=0.007). Flow cytometry analysis of MT Apoe−/− mice injected with Apoe−/− B cells to before Western diet feeding also revealed a significant decrease in aortic macrophage number (Figure 6I and 6J).

Discussion

The present study clearly demonstrates that B cells can attenuate atherosclerosis and provides the first evidence linking resident aortic B cells with this atheroprotection. Previous studies using transplantation of bone marrow from B-cell–deficient Ldlr−/− mice16 or splenectomy of Apoe−/− mice15 had suggested an atheroprotective role for B cells. However, more recent studies using a strategy of reducing circulating B cells with CD20 monoclonal antibody treatment resulted in attenuation of atherosclerosis,27,28 suggesting that the impact of B cells on atherosclerosis may be subset and context-dependent. Indeed, recent studies have

Figure 5. Id3−/− Apoe−/− B cells have decreased expression of CCR6 and expression of CCR6 on B cells mediates homing to the aorta and atheroprotection. A, Real-time PCR (see Methods for sequences) of B-cell mRNA from Id3−/− Apoe−/− mice expressed relative to Id3+/− Apoe−/− values. B, CCR6 staining of B cells from Id3+/− Apoe−/− or Id3−/− Apoe−/− mice. C, Percent specific migration of B cells in response to 500 ng/mL of CCL20 or 1000 ng/mL of CXCL13.*P=0.006. D, Luciferase activity from BJAB cells cotransfected with 2.3-kb human CCR6 promoter-luciferase construct and a pEF4 E12, empty pEF4, or pEF4 Id3 expression vectors. Experiments performed in triplicate were repeated 3 times. E, Representative flow plot, and F, quantification of B-cell staining within aortas harvested from 10- to 12-week-old C57BL/6 (B6) or Ccr6−/− mice (n=5) *P=0.032. G, Representative flow plot, and H, quantification of B-cell staining within aortas of Apoe−/− mice 24 hours after adoptive transfer of 60×10⁶ splenic B cells from B6 or Ccr6−/− mice or vehicle control. Each point represents a single animal. *P=0.009. I, Quantification of en face lesion area of MT Apoe−/− mice after adoptive transfer of 60×10⁶ B cells from either Ccr6−/− Apoe−/− or Ccr6−/− Apoe−/− mice or vehicle control. Animals were fed a Western diet for 16 weeks starting 72 hours after adoptive transfer. Each point represents a single animal. *P=0.0002, **P=0.002. NS indicates not significant.
addressed the subset question and provide evidence that B2 cells promote\textsuperscript{27,28} whereas B1a cells attenuate Western diet-induced atherosclerosis.\textsuperscript{29} Results herein, address the question of context, providing the first evidence that mice with B cells resident in the aorta at baseline have less atherosclerosis in response to Western diet feeding compared with those with few aortic B cells. \textit{Id3}\textsuperscript{-/-} Apoe\textsuperscript{-/-} mice, which had preserved numbers of circulating B cells but lacked sufficient aortic B cells (Figure 2F and 2G), developed significantly more atherosclerosis than \textit{Id3}\textsuperscript{+/-} Apoe\textsuperscript{-/-} mice (Figure 1).
Although it is possible that in addition to B cells, other cell types could contribute to the increased atherosclerosis observed in the \( \text{Id3}^{+/+} \text{Apoe}^{-/-} \) mouse, the use of the \( \mu \text{MT Apoe}^{-/-} \) recipient mouse in B-cell adoptive transfer studies demonstrates a clear role for Id3 in mediating B-cell homing and atheroprotection, as in the \( \mu \text{MT Apoe}^{-/-} \) model Id3 is present in all cell types. Adoptive transfer of splenic B cells from \( \text{Id3}^{-/-} \text{Apoe}^{-/-} \) mice led to equal reconstitution of peripheral lymph tissue (spleen, lymph node, and blood) as B cells from \( \text{Id3}^{+/+} \text{Apoe}^{-/-} \) mice (Table 2), but aortic reconstitution (Figure 3b) and attenuation of Western diet-induced atherosclerosis (Figure 4C) were significantly impaired. That the adoptively transferred B cells come from a hyperlipemic mouse on an atherogenic background (\( \text{Apoe}^{-/-} \)) appears to be important as neither 5\( \times \)10\(^8 \) or 6\( \times \)10\(^8 \) million B cells from B6 mice were atheroprotective when transferred into \( \mu \text{MT Apoe}^{-/-} \) recipient mice. Consistent with these findings, Western diet feeding induced Id3 expression in B cells (data not shown).

Further support for aortic B-cell–mediated atheroprotection is provided by the finding that B cells from \( \text{Apoe}^{-/-} \) mice null for CCR6 but wild-type for Id3 also had reduced aortic B-cell homing and B-cell–mediated atheroprotection (Figure 5). Taken together, results provide evidence for a model of atheroprotection whereby resident aortic adventitial B cells serve as early responders to atherogenic signals in the vessel wall to limit atherosclerosis.

Chemokine receptors provide the “address” for leukocyte homing to tissues and results of the present study provide the first evidence that CCR6 is important for B-cell trafficking to the aorta and attenuation of Western diet–induced atherosclerosis. Yet, mice globally deficient in CCR6 have attenuated atherosclerosis. CCR6 is expressed on various cell types involved in atherosclerosis, and mice globally deficient in CCR6 have reduced circulating monocytes due to an reduced ability of these cells to leave the bone marrow, providing evidence that CCR6 may have opposing effects on atherosclerosis development dependent on the cell type in which it is modulated. CCR6 has been identified as an important component of B-cell trafficking to form isolated lymphoid follicle formation in the gut in inflammatory states suggesting that CCR6 may be a common chemokine receptor mediating B-cell homing to tissue sites to modulate local inflammation. Results of the present study demonstrate that CCR6 is an Id3 target and that CCR6 regulates B-cell homing to the aorta and B-cell–mediated atheroprotection. Of note, the magnitude of the aortic homing defect in B cells from \( \text{Id3}^{-/-} \text{Apoe}^{-/-} \) mice is greater than that seen in \( \text{CCR6}^{-/-} \) mice, suggesting that additional chemokine receptors may participate with CCR6 in the aortic “address.”

Previous reports have identified CXCR6 and L-selectin as important regulators of T cell homing to the aorta, however our results suggest that the “address” for B-cell homing to the aorta is independent of these factors. Gräbner et al have implicated vessel wall expression of CXCL13 and CCL21 in the formation of B-cell–rich ATLOs adjacent to advanced plaques in aged \( \text{Apoe}^{-/-} \) mice. Yet, in our studies of younger mice, B cells from \( \text{Id3}^{+/+} \text{Apoe}^{-/-} \) and \( \text{Id3}^{-/-} \text{Apoe}^{-/-} \) mice had similar expression of CXCR5 (receptor for CXCL13) and CCR7 (receptor for CCL21) and transmigration in response to CXCL13 compared with controls (Figure 5 and data not shown), suggesting that unique chemokine signals may recruit B cells to the vessel wall before lesion development and in the early stages of atherosclerosis as compared with advanced lesions. It is also possible that Id3 may regulate other downstream target genes not involved in B-cell homing but involved in B-cell–mediated atheroprotection.

Id3 dimerizes with E-proteins such as the E2A gene products, E12 and E47, which have critical functions in B cells. As such, B cells from \( \text{Id3}^{-/-} \) mice may have other functional defects that promote atherogenesis. B cells produce antibodies and cytokine that modulate innate and adaptive immune responses. Evidence suggests that antibodies directed at oxidized phospholipid epitopes, such as OxLDL and MDA-LDL, may modulate atherosclerosis. In particular, IgM specific for OxLDL and MDA-LDL have been suggested to attenuate the development of atherosclerosis whereas IgG specific for these epitopes may be atherogenic. Given that the major site of LDL oxidation is in the artery wall, it seems plausible that resident aortic B cells may be stimulated with vessel wall antigens to produce protective autoantibodies in the spleen or local tissue. We therefore explored circulating levels of several of these antibodies known to be associated with atherosclerosis. Paradoxically, serum levels of IgM to MDA-LDL and OxLDL were both increased in \( \text{Id3}^{-/-} \text{Apoe}^{-/-} \) mice compared with control. Consistent with previous literature examining antibody levels in young \( \text{Id3}^{-/-} \) mice, the loss of Id3 in \( \text{Apoe}^{-/-} \) mice was also associated with an increase in total serum IgM levels. This raises the possibility that the increased IgM MDA-LDL and OxLDL observed in these mice may be secondary to a nonspecific global increase in all IgMs. Moreover, the increased, not decreased, levels of the putatively protective IgM MDA-LDL and OxLDL in the \( \text{Id3}^{-/-} \text{Apoe}^{-/-} \) mice make it unlikely that production of these specific antibodies is the mechanism for the increased atherosclerosis.

B cells have long been known to reside in the adventitia of diseased arteries of mice and humans, yet the specific location of these adventitial B cells in relation to atheroprone regions of the vascular tree has not been known. We demonstrate that B cells traffic to (Figure 3D) and reside (Figure 2G) in atheroprone regions of the aorta (the aortic arch and the descending abdominal aorta). Moreover, we demonstrate that B cells traffic to regions with existing lipid deposition (Figure 6A), reduce macrophage content within lesions (Figure 6F, 6G, and 6H) and retard the progression of these early lesions (Figure 6C). Interestingly, our results suggest that B cells regulate macrophage content of the aorta in response to Western diet feeding and even before feeding. Although \( \mu \text{MT Apoe}^{-/-} \) mice do not have B cells in their aortas, they do have abundant macrophages. Reconstitution of the aorta of \( \mu \text{MT Apoe}^{-/-} \) mice with \( \text{Id3}^{+/+} \text{Apoe}^{-/-} \) B cells resulted in a significant reduction in aortic wall–associated macrophages (Figure 6i and j). This significant alteration of the aortic cellular milieu did not occur when \( \text{Id3}^{-/-} \text{Apoe}^{-/-} \) B cells were used (data not shown), suggesting that the effect is dependent on aortic B-cell number. Notably, 8-week-old
chow-fed Id3<sup>−/−</sup> Apoe<sup>−/−</sup> mice, which have fewer aortic B cells, were also found to have more aortic macrophages (Figure 2). The number of cells in the aortas of Id3<sup>−/−</sup> Apoe<sup>−/−</sup> and adoptively transferred μMT Apoe<sup>−/−</sup> mice cannot be directly compared because these are distinct animal models and there is a significant difference in the degree of aortic B-cell deficiency as well as in the chronicity of the B-cell deficiency. However, both models independently provide evidence that B cells regulate aortic macrophage content. Given the rapid rate at which these changes occur, it is appealing to hypothesize that this effect is cytokine-mediated. Functional conduits connecting B-cell–containing ATLOs in the adventitia and the vessel wall have recently been identified<sup>5</sup>, suggesting that adventitial B cells may pass signals into the vessel wall. Immunoglobulins are too large to pass through these conduits<sup>5</sup>, however these conduits do allow passage of low-molecular-weight molecules such as cytokines from the ATLO to the media. Thus, adventitial B cells could limit macrophage accumulation in the intima through local production of anti-inflammatory cytokines that can pass into the vessel wall via these conduits. Future studies will be necessary to confirm these hypotheses and identify the cytokines involved.

The Id3<sup>−/−</sup> Apoe<sup>−/−</sup> mouse provides a unique model with which to explore the cellular and molecular atheroprotective mechanisms of leukocytes resident in the aortic adventitia of nondiseased vessels. Using this model, our findings provide evidence for a model whereby resident adventitial B cells within this microenvironment are poised to react to atherogenic stimuli from the vessel wall leading to production of factors that limit macrophage accumulation in plaque and progression of lesion development. Given the previously identified association between a functionally significant SNP in the human ID3 gene and carotid intima medial thickness, our findings in mice may also have implications for human disease. Thus, identification of Id3 as a critical regulator of aortic B-cell homing and atheroprotection is not only important for our understanding of these mechanisms but may also lead to novel strategies to attenuate atherosclerosis in humans.

Acknowledgments

We thank Hong Pei and the UVA Mouse Surgery Transplantation and Physiology Core for excellent technical assistance, Jeremy Gatesman from the UVA Center for Comparative Medicine for excellent technical assistance, Dr Borna Mehrad (UVA) for generously providing Id3<sup>−/−</sup>, and Mike Solga from the UVA Flow Cytometry Core for excellent advice.

Sources of Funding

This work was supported by NIH P01 HL55798 (C.A.M.), NIH R01 HL62522 (C.A.M.), NIH R01 HL096447 (C.A.M.), NIH R01 HL58108 (K.L.), R01 HL08559 (J.L.W. and S.T.), P01 HL088093 (J.L.W. and S.T.), NIH ARRA HL096447-01A1 (S.T.), Fondation Leducq (S.T. and J.L.W.), NIH Training Grant 5-T32 HL007355-29 (M.J.L. and A.C.), American Heart Association Pre-Doctoral Fellowship (A.C.D.), and American Heart Association Mid-Atlantic Affiliate postdoctoral fellowship award 10POST3560000 (M.J.L.).

Disclosures

None.

References

What New Information Does This Article Contribute? 

- B cells have been shown to both aggravate and attenuate atherogenesis, suggesting that the effects of B cells may be subset- or context-dependent. Here, we provide evidence that the effects of B cell on atherosclerosis are context-dependent based on the following findings: (1) ApoE<sup>−/−</sup> mice with reduced number of aortic, but normal number of peripheral B cells have increased atherosclerosis. (2) B cells home to, and attenuate initiation and progression of atherosclerosis in, regions of the aorta prone to atherosclerosis and attenuate initiation and progression of atherosclerosis. Our results identify the helix-loop-helix factor, ID3 as a critical regulator of B-cell aortic homing and B-cell-mediated atheroprotection and define CCR6 as one downstream target of ID3 that promotes aortic B-cell homing and B-cell-mediated atheroprotection. Taken together with previous findings of a functionally significant SNP in the human ID3 gene associated with carotid thickening in humans, these results suggest that strategies to enhance ID3 expression or function may provide immune protection against atherosclerosis.
B-Cell Aortic Homing and Atheroprotection Depend on Id3

Amanda C. Doran, Michael J. Lipinski, Stephanie N. Oldham, James C. Garmey, Kirsti A. Campbell, Marcus D. Skaflen, Alexis Cutchins, Daniel J. Lee, David K. Glover, Kimberly A. Kelly, Elena V. Galkina, Klaus Ley, Joseph L. Witztum, Sotirios Tsimikas, Timothy P. Bender and Coleen A. McNamara

Circ Res. published online October 27, 2011;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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**SUPPLEMENTAL MATERIALS**

**Supplementary Table I.** Average random plasma lipid, oxidized lipoprotein, immunoglobulin, glucose and insulin profiles of *Id3*+/+ *Apoe*−/− and *Id3*−/− *Apoe*−/− mice at baseline (eight weeks of age).

<table>
<thead>
<tr>
<th></th>
<th><em>Id3</em>+/+ <em>Apoe</em>−/−</th>
<th><em>Id3</em>−/− <em>Apoe</em>−/−</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>24.40 ± 2.19</td>
<td>23.10 ± 3.34</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>353 ± 53</td>
<td>355 ± 32</td>
<td>n.s.</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>32 ± 5</td>
<td>34 ± 9</td>
<td>n.s.</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>187 ± 44</td>
<td>179 ± 40</td>
<td>n.s.</td>
</tr>
<tr>
<td>Non-HDL (mg/dL)</td>
<td>311 ± 46</td>
<td>315 ± 40</td>
<td>n.s.</td>
</tr>
<tr>
<td>OxPL/ApoB</td>
<td>2.42 ± 0.69</td>
<td>2.54 ± 0.50</td>
<td>n.s.</td>
</tr>
<tr>
<td>MDA/ApoB</td>
<td>4.54 ± 1.33</td>
<td>3.13 ± 0.47</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total IgM (μg/ml)</td>
<td>100 ± 25</td>
<td>275 ± 60</td>
<td>0.038</td>
</tr>
<tr>
<td>MDA LDL IgM (RLU)</td>
<td>7,044 ± 1,567</td>
<td>15,924 ± 3,045</td>
<td>0.021</td>
</tr>
<tr>
<td>Cu-OxLDL IgM (RLU)</td>
<td>2,124 ± 281</td>
<td>3,722 ± 539</td>
<td>0.028</td>
</tr>
<tr>
<td>MDA LDL IgG (RLU)</td>
<td>1,308 ± 280</td>
<td>2,501 ± 711</td>
<td>n.s.</td>
</tr>
<tr>
<td>Cu-OxLDL IgG (RLU)</td>
<td>355 ± 42</td>
<td>584 ± 81</td>
<td>n.s.</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>135 ± 25</td>
<td>139 ± 21</td>
<td>n.s.</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.56 ± 0.21</td>
<td>0.57 ± 0.26</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values are the average ± SEM
n.s.: not significant

**Supplementary Table II.** Average random plasma lipid, glucose and insulin profiles of bone marrow transplant chimeric mice.

<table>
<thead>
<tr>
<th></th>
<th><em>Id3</em>+/+ <em>Apoe</em>−/−</th>
<th><em>Id3</em>−/− <em>Apoe</em>−/−</th>
<th><em>Id3</em>+/+ <em>Apoe</em>−/−</th>
<th><em>Id3</em>−/− <em>Apoe</em>−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>25.73 ± 3.32</td>
<td>25.00 ± 4.18</td>
<td>23.70 ± 2.67</td>
<td>24.64 ± 2.25</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>1,587 ± 164</td>
<td>1,598 ± 194</td>
<td>1,583 ± 123</td>
<td>1,559 ± 152</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>23 ± 3</td>
<td>26 ± 4</td>
<td>21 ± 4</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>211 ± 34</td>
<td>198 ± 28</td>
<td>204 ± 41</td>
<td>201 ± 32</td>
</tr>
<tr>
<td>Non-HDL (mg/dL)</td>
<td>1,523 ± 148</td>
<td>1,532 ± 134</td>
<td>1,526 ± 145</td>
<td>1,499 ± 118</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>177 ± 29</td>
<td>160 ± 9</td>
<td>154 ± 19</td>
<td>162 ± 25</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>2.81 ± 0.94</td>
<td>3.10 ± 0.81</td>
<td>3.04 ± 0.78</td>
<td>2.99 ± 0.74</td>
</tr>
</tbody>
</table>

Values are the average ± SEM
No significant difference in any parameter between groups.
METHODS SUPPLEMENT

Animals

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. C57BL/6, Apoe<sup>-/-</sup> and µMT mice were purchased from Jackson Laboratory (stock #000664, #002052 and #002288 respectively). Id3<sup>-/-</sup> mice were a generous gift of Dr. Yuan Zhang (Duke University). Id3<sup>-/-</sup> and µMT mice were bred to the Apoe<sup>-/-</sup> background to obtain atherogenic Id3<sup>-/-</sup> Apoe<sup>-/-</sup> and µMT Apoe<sup>-/-</sup> strains. Ccr6<sup>-/-</sup> mice were a generous gift from Dr. Borna Mehrad (University of Virginia). All mice were given water ad libitum and either a standard chow diet (Harlan Teklad catalog #7012) or Western diet (Harlan Teklad catalog #TD88137). Only male mice were used for all experiments.

Determination of Serum Cholesterol Levels and Antibody Titers

Whole blood was harvested from mice at the time of sacrifice by right ventricular puncture. Blood was centrifuged at 8200 rcf (10,000 rpm) for five minutes, after which the serum layer was removed to a clean tube. Cholesterol determinations were performed by the University of Virginia Medical Laboratories using an Archtect 8000 series analyzer, employing the method of Allain<sup>1</sup> and Roeschla<sup>2</sup>. Determination of mouse apoB levels, malondialdehyde epitopes on mouse apoB, and mouse IgG and IgM MDA-LDL and Cu-OxLDL were determined as previously described in detail<sup>3,4</sup>.

Analysis of Atherosclerosis

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine and the vasculature was perfused by left ventricular puncture using 4% paraformaldehyde. Aortas were opened longitudinally, pinned and stained using Sudan IV by the en face method as previously described<sup>5</sup>. Aortas were then imaged and lesion area was quantified using ImagePro 5.0. In the experiments in which B cells were adoptively transferred to µMT Apoe<sup>-/-</sup> mice already fed Western diet for 8 weeks, the heart and aortic arch to the left subclavian artery was embedded in paraffin and 5 μm thick serial sections were generated from the aortic valve to the bifurcation of the brachiocephalic artery in each animal. Sections were stained at 60 μm intervals using the Movat method<sup>6</sup> and plaque volume was assessed using ImagePro Plus 7.0 software. The descending aortas from the left subclavian artery to the iliac bifurcation were opened longitudinally, pinned and stained using Sudan IV as described above.

Immunohistochemical Analysis of Atherosclerosis

Paraffin-embedded sections were selected at 60 μm intervals starting at the aortic valve, deparaffinized, rehydrated, underwent antigen retrieval, blocked with PBS with fish skin gelatin and 10% horse serum for 1 hour, incubated with the primary antibody for 1 hour, washed and then incubated with the secondary antibody for 1 hour. When staining for macrophages, the primary antibody was applied at a concentration of 1:250 (Rat anti-mouse Mac-2 monoclonal IgG antibody, Cedarlane Laboratories) and the secondary antibody was applied at a concentration of 1:100 (Dylight 594-conjugated donkey anti-rat IgG antibody, Jackson ImmunoResearch Laboratories). When staining for MCP-1, the primary antibody was applied at a concentration of 1:250 (Goat anti-mouse MCP-1 polyclonal IgG antibody, Santa Cruz Biotechnology Inc, Cat # sc-1784) and the secondary antibody was applied at a concentration of 1:250 (Dylight 488-conjugated donkey anti-goat IgG antibody, Jackson ImmunoResearch Laboratories). The slides were then washed and the coverslip was placed following application of Vectashield Mounting Medium with DAPI (Vector Laboratories). Negative controls were also performed in which the slides were processed as above with omission of the primary antibody for one control and the secondary antibody for another control. Fluorescent microscopy was
performed along with acquisition of brightfield images. Macrophage area / intima area was calculated as the macrophage area within the intima divided by the total area of the intima using ImagePro Plus 7.0 software. MCP-1 area / intima area was calculated as the MCP-1 area within the intima divided by the total area of the intima.

**Bone Marrow Transplantation**

At four to five weeks of age, *Id3*+/+*Apoe*−/− and *Id3*−/−*Apoe*−/− mice were subjected to a sub-lethal dose of radiation (500 rads x 2 irradiations) and subsequently reconstituted with 5 x 10⁶ bone marrow cells harvested from the femurs and tibias of *Id3*+/+*Apoe*−/− and *Id3*−/−*Apoe*−/− donor mice. After four weeks of recovery (eight weeks of age), recipient animals were started on a Western diet. After 16 weeks of Western diet, the aortas were harvested and analyzed by the en face method described above.

**FACS Analysis of Murine Aortas**

A version of this method as well as all antibodies used for these studies have been previously validated and published. Animals were lethally anesthetized by intraperitoneal injection of ketamine/xylazine and the vasculature was perfused by left ventricular puncture using PBS with 20 U/ml heparin. The aorta was microdissected, taking great care to remove all periaortic fat and lymph nodes while sparing the adventitia as this is where B cells are predominantly found. Lymph nodes, spleens, blood and aortas, including the adventitia, were harvested under a dissection microscope. Tissues were homogenized and incubated for 45 minutes at 37°C in an enzymatic cocktail (1ml per 10mg tissue) containing 488 U/ml collagenase I (Sigma cat # C0130), 230 U/ml collagenase XI (Sigma cat # C7657), 125 U/ml hyaluronidase (Sigma cat # H3506) and 60 U/ml DNase I (Sigma catalog # D4527) in an enzymatic digestion buffer containing PBS and 20mM HEPES. Tissues were then mashed through a 70-μm cell strainer to create a suspension. Red blood cells were lysed from spleen and blood samples using a lysis buffer containing 155mM NH₄Cl, 10 mM KHCO₃ and 0.1mM EDTA. Cells were counted, diluted to 1 x 10⁶ cells per 100 μl and incubated with antibodies (as named above) for 45 minutes at 4°C. After washing, the cells were then fixed, permeabilized and stained with antibodies directed against specific intracellular antigens using the Caltag Fix & Perm kit as per the manufacturer’s instructions. Cells were washed again, resuspended in FACS buffer (PBS containing 0.05% NaN₃ and 1% BSA) and analyzed at the University of Virginia Flow Cytometry Core using a CyAN ADP flow cytometer. Data were analyzed using FloJO software (Tree Star Inc).

**FACS Antibodies**

Antibodies used were as follows: anti-CD3e (APC, clone 145-2C11, 0.2 μg used at 1:100), anti-CD5 (PE, clone 53-7.3, 0.1 μg used at 1:200), anti-CD11b (Pacific Blue, clone M1/70/15, 0.1 μg used at 1:100), anti-CD11c (APC AlexaFluor750, clone N418, 0.4 μg used at 1:50), anti-CD16/32 (purified Fc Block, 0.5 μg used at 1:100), anti-CD19 (PECy7, clone 1D3, 0.2 μg used at 1:100), anti-CD21 (APC, clone 7G6, 0.2 μg used at 1:100), anti-CD23 (PECy7, clone B3B4, 0.2 μg used at 1:50), anti-CD45 (PerCP, clone 30-F11, 0.2 μg used at 1:100), anti-CD45R/B220 (PE, clone RA3-6B2, 0.2 μg used at 1:100), anti-CD68 (FITC, clone FA-11, 0.5 μg used at 1:50), F4/80 (APC, clone A3-1, 0.05 μg used at 1:100), Gr-1 (APC Cy7, clone RB6-8C5, 0.2 μg used at 1:100), I-A<sup>β</sup> (FITC, clone AF6-120.1, 0.5 μg used at 1:100), anti-IgD (FITC, clone 11-26, 0.5 μg used at 1:200), anti-IgM (APC, clone II/41, 0.2 μg used at 1:50), anti-NK1.1 (PECy7, clone PK136, 0.2 μg used at 1:100), anti-TCR<sub>β</sub> (APC AlexaFluor750, clone H57-597, 0.2 μg used at 1:50), anti-Ter119 (FITC, 0.5 μg used at 1:100). Either 7-AAD (BD Pharmingen) or LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) was included as a viability stain with every sample.
Adoptive Transfer

Spleens were harvested from 10-12 week old animals and B cells were isolated using MACS anti-CD43 microbeads (Miltenyi Biotec) as per the manufacturer’s protocol. The purity of representative samples were analyzed by flow cytometry and found to be greater than 98%. Either 30 $\times$ 10$^6$ or 60 $\times$ 10$^6$ B cells were suspended in 200 µl of PBS and adoptively transferred via tail vein injection to 8-10 week old recipients.

Fluorescent Microscopy of Aortic Cross Sections

B lymphocytes were purified as above from the spleens of $Id3^{+/+}$ $Apoe^{-/-}$ mice. Cells were incubated in 10 µM CFDA-SE, prepared as per the manufacturer’s instructions, for 30 minutes (Invitrogen, catalog # V12883). Lymphocytes were then washed and adoptively transferred via tail vein injection to $\mu$MT $Apoe^{-/-}$ recipients. After 72 hours, mice were euthanized, aortas were harvested and embedded into OCT medium. After freezing at -20°C for one hour, samples were cut into 50µm thick sections using a cryostat and mounted onto glass slides with DAPI-containing medium. Fluorescent imaging at the UVA Advanced Microscopy Core enabled identification of B cells using standard set green channel filters to detect CFDA-SE positive cells and the blue channel for DAPI.

Optical Imaging of Aortic B Lymphocytes

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine, the vasculature was perfused by left ventricular puncture using phosphate buffered saline and the aorta was carefully removed by microdissection. As with dissection for flow cytometric analysis, great care was again taken to remove all periaortic fat and lymph nodes while sparing the adventitia in order to preserve aortic lymphocytes. In order to detect the endogenous presence of B lymphocytes in the aorta with near-infrared (NIR) fluorescent imaging, 1 ml of a biotinylated anti-B220 antibody (eBioscience catalog # 14-0452, used at 0.5 mg/ml) and molar excess of Cy5.5-conjugated streptavidin (Rockland Immunochemicals, catalog # S000-13) were mixed and incubated for 30 minutes to ensure adequate conjugation. Aortas were then harvested as described above from five chow-fed eight to ten week-old $Id3^{+/+}$ $Apoe^{-/-}$ mice, five $Id3^{-/-}$ $Apoe^{-/-}$ mice, and five $\mu$MT $Apoe^{-/-}$ mice. Each aorta was incubated overnight at 5°C in 65 µL of Cy5.5-labeled anti-B220 solution diluted in 335 µL of PBS while rotating in the dark in amber Eppendorf tubes. The aortas were then washed three times with PBS for ten minutes and the aortic lumen was flushed with PBS to minimize non-specific retention of fluorescent antibody. Ex vivo fluorescence-mediated tomography (FMT) quantitative imaging (FMT 2500, VisEn Medical) was performed for each aorta with data acquisition at 680 nm for excitation and 700 nm for emission. Regions of interest were generated for each aorta and values for mean fluorescence from 2-dimensional fluorescence reflectance images were obtained. To enable comparison between the aortas, the maximal fluorescence was set to 10.5 count/energy for the NIH Color scale bar.

Radiolabeling of B Lymphocytes

Ten to 12 week-old $Id3^{-/-}$ $Apoe^{-/-}$ and $Id3^{+/+}$ $Apoe^{-/-}$ mice were euthanized with carbon dioxide, the spleens were removed and a single cell suspension was obtained by pressing the spleen through a 70-µm strainer. Red blood cells were lysed from the cell suspension using a lysis buffer containing 155mM NH4Cl, 10 mM KHCO3 and 0.1mM EDTA. B lymphocytes were isolated using MACS anti-CD43, anti-CD11b, and anti-CD4 microbeads (Miltenyi Biotec) as per the manufacturer’s protocol. This combination of beads resulted in a B cell purity of 99% on flow cytometry. Molar excess of oxyquinolone (oxine) was incubated with indium-111 HCl (PerkinElmer) for 30 minutes at room temperature in HEPES Buffer with 10% dimethyl sulfoxide, enabling formation of indium-111 oxine. Indium-111 has a half-life of 2.8 days with
photon energies of 171 and 245 keV. Isolated B lymphocytes were centrifuged and resuspended with indium-111 oxine solution and allowed to incubate at room temperature for one hour. Cells were washed three times with normal saline after which B lymphocyte number and percent cellular viability was then determined by counting cells following Trypan Blue staining.

The radiolabelled B lymphocytes from either Id3+/+ Apoe−/− or Id3−/− Apoe−/− mice were then adoptively transferred via tail vein injection into μMT Apoe−/− recipient mouse. The μCi of indium-111 per injected dose was determined by measuring the radioactivity of the syringe before and following injection into the mouse using a Radioisotope Dose Calibrator (Searle Radiographics). Additionally, control μMT Apoe−/− recipient mice received an equal total radioactive dose of indium-111 oxine diluted in normal saline, thus lacking radiolabeled cells.

**Ex Vivo Nuclear Imaging of Radiolabeled B Lymphocytes**

Approximately 20 hours following adoptive transfer of radiolabeled B lymphocytes or control injection, the recipient mice were euthanized by intraperitoneal injection of ketamine/xylazine, the vasculature was perfused with PBS, the aortas were harvested whole from just above the heart to the iliac bifurcation as previously described, cut longitudinally and opened en face, covered with plastic saran wrap to minimize dehydration, and exposed to a high sensitivity, medium resolution phosphor imaging screen (PerkinElmer). After 18 hours, the phosphor imaging screen was scanned using a PerkinElmer Cyclone Plus Phosphor Imaging System. Signal intensity analysis for each aorta was performed using Sante DICOM software and was adjusted for injected radioactive dose per animal and background using the formula: (Mean Signal Intensity of Aorta - Background Signal Intensity) / Injected Radioactive Dose in μCi. Regions of interest within the aorta were defined by anatomical landmarks. The aortic arch was defined as the region from the aortic cusp to the left subclavian artery. The descending thoracic aorta was defined as the region from the left subclavian artery to the diaphragm. The descending abdominal aorta was defined as the region from the diaphragm to the bifurcation of the iliac arteries. Comparison of aortic indium-111 content for recipient aortas was then determined using a Minaxi 5500 Gamma Counter using an indium-111 window setting (160 to 485 keV). Gamma counting for each sample occurred over a five minute period to generate an average number of counts per minute (CPM) and the adjusted gamma counts for each aorta are determined by the formula: ((CPM of aorta - Background CPM) / Weight of the aorta in grams / Injected radioactive dose in μCi).

**Real Time PCR**

Total cellular RNA was collected from B cells using an RNeasy kit (Qiagen) as per the manufacturer’s instructions. cDNA was then synthesized using an iScript cDNA synthesis kit (BioRad). Total cDNA was diluted 1:5 in water and 2 μl were used for each real-time PCR reaction using a Bio-Rad iCycler and iQ SYBR Green Supermix (BioRad). Analysis was performed by normalizing to cyclophilin using the standard curve method to approximate the amount of starting material. Primers used for the detection of CCR6 were: 5′ - CCC AGC ACA TCA TAG CAT TG - 3′ and 3′ - AGT CCG AAA GAC CCA GGA TT - 5′. Primers for the detection of CXCR5 were: 5′ - AGG AAA ACG AAG CGG AAA CT - 3′ and 3′ - CGA CCA GCA GAG GAA GAA AA - 5′. Primers for the detection of CXCR6 were: 5′ - AAC AGC CAG GAG AAC AAA CG - 3′ and 3′ - ACA CAC AGG GCA AAA AGA CC - 5′. Primers for the detection of L-selectin were: 5′ - AGC CCA ACA ACA AGA AGT CC - 3′ and 3′ - TCG TTC CAT TTC CCA GAG TC - 5′. Primers for the detection of cyclophilin were: 5′ - TGC CGG AGT CGA CAA TGA T - 3′ and 3′ - TGG AGA GCA CCA AGA CAG ACA - 5′.
B cell Migration
Splenic B cells were purified from Id3+/+ Apoe−/− and Id3−/− Apoe−/− mice as described above and resuspended in RPMI 1640 (Invitrogen) with 10% FBS (HyClone). 1 x 10^6 cells were placed in the upper chamber of 5 μm pore size transwells containing either 1000 ng/ml of CXCL13 or 500 ng/ml of CCL20 in the bottom of the transwell. After incubating for 6 hours, the number of cells which had migrated through the transwell were counted using a flow cytometer. The percent specific migration was determined by subtracting the spontaneous migration observed in transwells with no chemokine from the migration observed under experimental conditions.

Statistical Methods
Data are presented as the mean ± standard error of the mean. Unpaired Student’s t test, Mann Whitney U-test, or analysis of variance was performed to compare continuous variables between groups (presented as mean ± standard error of the mean (SEM)). Bonferroni’s correction was applied to the analysis of variance when comparisons between multiple groups were performed to provide comparison between all groups. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using NCSS 2001 (Number Crunching Statistical Software, Kaysville, Utah) and GraphPad Prism5 (La Jolla, California).

References
6. Movat HZ. Demonstration of all connective tissue elements in a single section; pentachrome stains. AMA Arch Pathol. 1955;60:289-95.