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B Cell Aortic Homing and Atheroprotection Depend on Id3

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Abstract

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 micereconstituted 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.

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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.

Keywords

Atherosclerosis; B lymphocytes; Transcription factors; Helix-loop-helix; Homing

INTRODUCTION

Atherosclerosis is a progressive, chronic inflammatory disease resulting in plaque formation in large arteries[1, 2] that can lead to heart attack, stroke and death. Targeting the immune cells that participate in atherogenesis is a promising novel approaches 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, while ATLOs are not present in the normal/non-inflamed aorta prior to 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 prior to Western diet feeding[15, 16] results in increased atherosclerosis in mice, suggesting 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 dominant 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's-like syndrome with lachrymal and salivary glands lymphocytic infiltrates[18, 19], 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 wildtype 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 (cIMT) in humans[21].

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 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 10–12 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 Archtect 8000 series analyzer. Antibody titers were determined as detailed in the 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 utilized 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 Biotechnology Inc) and macrophage content was determined with Mac-2 staining (Cedarlane Laboratories). These protocols are detailed in the Supplemental Material.

Bone Marrow Transplantation

$Id3^{+/+}Apoe^{-/-}$ and $Id3^{-/-}Apoe^{-/-}$ mice were subjected to a sub-lethal 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 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 described[14] and detailed in the Supplemental Material.

Ex Vivo Imaging of Radiolabeled B cells

B cells were radiolabeled by incubation in indium-111 oxine solution. Following 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 Supplemental Material.

Optical Imaging of Aortic B Cells

Ex vivo fluorescence-mediated tomography (FMT) quantitative imaging (FMT 2500, VisEn Medical) was performed following incubation of aortas with Cy5.5-labeled anti-B220 antibodies (eBiosciences and Rockland Immunochemicals). This protocol is detailed in the Supplemental Material.

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), and real-time PCR reaction using a Bio-Rad iCycler and iQ SYBR Green Supermix (BioRad) was performed as detailed in the 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 which had migrated through the transwell were counted using a flow cytometer as detailed in the Supplemental Material.

Statistical Analysis

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). Statistical analysis is detailed in the 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 Western diet for four or eight weeks, then harvested for en face analysis of lesion area (Figure 1a). After four weeks of Western feeding, *Id3*$^{-/-}$ *Apoe*$^{-/-}$ but not *Id3*$^{+/+}$ *Apoe*$^{-/-}$ mice developed a discernable amount of lesion (0.053% ± 0.009 vs 1.16% ± 0.483; p=0.004). After eight weeks of Western feeding, both genotypes developed detectable atherosclerosis, with significantly more lesion seen in the *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mouse (0.347% ± 0.111 vs 4.03% ± 0.859; p=0.002) (Figure 1b). There were no differences in body weight, serum lipids, or the content of immunochemically detected oxidized phospholipids (OxPL) or malondialdehyde (MDA) epitopes on apoB lipoproteins (oxPL/ApoB or MDA/apoB), glucose or insulin in *Id3*$^{-/-}$ *Apoe*$^{-/-}$ compared with *Id3*$^{+/+}$ *Apoe*$^{-/-}$ mice (Supplementary Table I).

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 to 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 1e). There was a significant increase in atherosclerosis when *Id3*$^{-/-}$ *Apoe*$^{-/-}$ compared to 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 chimeras (Supplementary Table II).

Id3$^{-/-}$ Apoe$^{-/-}$ Mice Have Significantly Fewer B Cells in the Aortas 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 prior to 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 f). 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, periaortic lymph nodes and whole blood were similar between *Id3*$^{+/+}$ *Apoe*$^{-/-}$ and *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice (Table 1).

Optical Imaging Confirms Fewer B Cells in Aorta of Id3$^{-/-}$ Apoe$^{-/-}$ 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 *Id3*$^{-/-}$ *Apoe*$^{-/-}$ and *Id3*$^{+/+}$ *Apoe*$^{-/-}$ mice. As a control, μMT mice, which lack peripheral B cells due to deletion of genomic DNA sequences that encode the transmembrane domain of the B cell receptor μ heavy chain[22], were bred to *Apoe*$^{-/-}$ mice to generate B cell-deficient *Apoe*$^{-/-}$ mice (μMT *Apoe*$^{-/-}$). B cell-deficient μMT *Apoe*$^{-/-}$ aortas were found to have a minimal amount of background signal, which likely represents the non-specific retention of the Cy5.5-labeled anti-B220 antibody. While a significant increase in fluorescence over background was observed in both *Id3*$^{+/+}$ *Apoe*$^{-/-}$ and *Id3*$^{-/-}$ *Apoe*$^{-/-}$ aortas (Figure 2g), consistent with our previous flow cytometric data, the increase in fluorescence over controls in the aortas of *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice was significantly lower than that in the aortas of *Id3*$^{+/+}$ *Apoe*$^{-/-}$ mice (15% vs 42%; $p<0.0006$) (Figure 2g and h). 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].

Id3 is Necessary for B Cell Homing to the Aorta

To determine if Id3 regulated homeostatic trafficking of B cells to the aorta, adoptive transfer studies were conducted. Splenic B cells from *Id3*$^{+/+}$ *Apoe*$^{-/-}$ or *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice were adoptively transferred to μMT *Apoe*$^{-/-}$ 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*$^{-/-}$ B cells wild-type for Id3 appear in the aorta six hours after tail vein injection and continue to accumulate in the aorta up to 72 hours later. In contrast, no appreciable numbers of *Id3*$^{-/-}$ *Apoe*$^{-/-}$ 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 aortic 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 intoμMT *Apoe*$^{-/-}$ mice was performed. Splenic B cells were isolated from *Id3*$^{+/+}$ *Apoe*$^{-/-}$ and *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice and radiolabeled with indium-111 (In-111) oxine. Recipient μMT *Apoe*$^{-/-}$ mice were injected with 1×10^7 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*$^{+/+}$ *Apoe*$^{-/-}$ compared with *Id3*$^{-/-}$*Apoe*$^{-/-}$ B cells (Figure 3c and d). Consistent with optical imaging data (Figure 2g), radiolabelled B cells traffic to regions of the aorta that are prone to atherosclerosis (Figure 3c and e). To determine the layer of the vessel wall to which B cells traffic in these experiments, splenic B cells were purified from *Id3*$^{+/+}$ *Apoe*$^{-/-}$ mice, incubated with CFDA-SE and adoptively transferred to μMT *Apoe*$^{-/-}$ mice. Seventy-two hours after transfer, aortas were harvested from mice, sectioned and analyzed for the presence of these labeled B cells. While 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.

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 via 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 vs 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 vs 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 vs 8.18% ± 0.735 or 7.82% ± 1.16) (Figure 4b and c). 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 prior to 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 the chemokine receptors on B cells that may be regulating homing to the aorta in an Id3-dependent manner, we performed a murine PCR chemokines and receptor array (SA Biosciences) using RNA from B cells derived from *Id3*$^{+/+}$ *Apoe*$^{-/-}$ or *Id3*$^{-/-}$ *Apoe*$^{-/-}$ 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 three-fold reduction in CCR6 mRNA expression in *Id3*$^{-/-}$ *Apoe*$^{-/-}$ as compared with *Id3*$^{+/+}$ *Apoe*$^{-/-}$ 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 to the aorta[14, 24]. Differences in surface expression of CCR6 protein was confirmed by flow cytometry (Figure 5b) and impaired migration of *Id3*$^{-/-}$ *Apoe*$^{-/-}$ B cells in response to the cognate ligand for CCR6 (CCL20) was demonstrated using transwell assays (Figure 5c). Id3 regulates target gene expression through dimerization with E2A gene products, like E12, antagonizing E12 DNA binding and transcription regulatory effects. Transient co-transfection studies in BJAB cells (a human B cell lymphoma line), demonstrated that E12 inhibits CCR6 promoter activation, an effect antagonized by co-transfection 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 f). 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 h). Moreover, adoptive transfer of 60 × 10^6 *Ccr6*$^{+/+}$*Apoe*$^{-/-}$ B cells to μMT *Apoe*$^{-/-}$mice significantly reduced atherosclerotic lesion area in response to 16 weeks of Western diet feeding (16.1% ± 0.992 vs 9.28% ± 0.783; p=0.0002). In contrast, lesion area was not significantly changed in animals receiving *Ccr6*$^{-/-}$ *Apoe*$^{-/-}$ B cells (16.1% ± 0.992 vs 13.4% ± 0.823; p=n.s.) (Figure 5i). These data identify CCR6 as an Id3 target involved in the aortic ‘address’ for B cell homing and B cell-mediated atheroprotection.

B Cells traffic to Regions of the Aorta with Existing Atherosclerosis

Optical and phosphor imaging demonstrated that B cells traffic to regions of the aorta known to be prone to atherosclerosis (Figure 2 and 3). To directly determine if B cells traffic to regions of atherosclerosis, μMT *Apoe*$^{-/-}$ mice were fed 8 weeks of Western diet to develop atherosclerosis. Animals were then injected with 2 × 10^7 radiolabeled *Apoe*$^{-/-}$ B cells or vehicle control. En face staining with Sudan IV demonstrated lipid deposition predominantly in the arch and abdominal aorta. *Ex vivo* phosphor imaging of aortas revealed the highest signal intensity in the regions of the aorta positive for Sudan IV staining (Figure 6a). Injection of an equal number of radiolabeled *Id3*$^{-/-}$ *Apoe*$^{-/-}$ B cells served as a control as we previously demonstrated an aortic homing defect in B cells null for Id3 (Figure 6a and b).

Delivery of Id3$^{+/+}$ Apoe$^{-/-}$ B Cells Inhibited Progression of Western diet-induced Atherosclerosis: An effect attenuated by loss of Id3

B cell injection significantly inhibited atherosclerosis development when the B cells were delivered prior to Western diet feeding[15, 16] (Figure 4). To determine whether B cells impact the progression of existing atherosclerosis, *Apoe*$^{-/-}$ B cells were adoptively transferred into μMT *Apoe*$^{-/-}$ mice with existing atherosclerosis. The μMT *Apoe*$^{-/-}$ mice fed 8 weeks of a Western diet underwent baseline atherosclerosis quantification (n=10) or received 45 × 10^6 *Apoe*$^{-/-}$ B cells (n=9) or vehicle control (n=13). Injected animals received an additional 8 weeks of Western diet and were then assessed by en face staining of the descending aorta and cross-sectional analysis of the aortic arch. Movat staining of cross sections confirm that en face lipid staining was accompanied by the histomorphologic components of atherosclerotic plaque (necrotic core and cellular infiltration). Consistent with whole aorta en face findings (Figures 4 and 6), there was significant atherosclerosis in the abdominal aorta by en face staining and in the arch by cross-sectional analysis in the vehicle treated group. In contrast, adoptive transfer of B cells from *Apoe*$^{-/-}$ mice significantly attenuated the progression of atherosclerosis when compared with vehicle recipients as measured by *en face* (4.9 ± 0.7% vs 9.7 ± 1.3% respectively, p=0.011) (Figure 6c) and cross-sectional analysis (35.6 ± 3.2% vs 45.3 ± 3.1%, p=0.046) (Figure 6d and e). Serum collected at the time of aorta harvest was analyzed for lipids. There were no significant differences in plasma cholesterol, triglycerides, or OxPL/apoB levels between the groups (data not shown).

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*$^{+/+}$ *Apoe*$^{-/-}$ 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 (Supplement Table I). Macrophages are present in the aorta prior to Western diet feeding[14] (Figure 2d) and infiltrate the vessel wall early in the disease process[3]. To determine if B cell delivery to μMT *Apoe*$^{-/-}$ mice alters plaque macrophage content, immunohistochemical staining of plaques from vehicle and B cell injected μMT *Apoe*$^{-/-}$ mice was performed. Compared to control, adoptive transfer of B cells resulted in a significant reduction in Mac2 (Figure 6f and g, 0.34 ± 0.02 vs. 0.42 ± 0.02 respectively, p=0.024), and MCP-1 staining (Figure 6f and h, 0.074 ± 0.009 vs. 0.121 ± 0.012, p=0.007). Flow cytometry analysis of μMT *Apoe*$^{-/-}$ mice injected with *Apoe*$^{-/-}$ B cells to prior to Western diet feeding also revealed a significant decrease in aortic macrophage number (Figure 6i and j).

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 utilizing transplantation of bone marrow from B cell-deficient μMT to *Ldlr*$^{-/-}$ mice[16] or splenectomy of *Apoe*$^{-/-}$ mice[15] 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 addressed the subset question and provide evidence that B2 cells promote[27, 28] while B1a cells attenuate Western diet-induced atherosclerosis[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 to those with few aortic B cells. *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice, which had preserved numbers of circulating B cells but lacked sufficient aortic B cells (Figure 2f and g), developed significantly more atherosclerosis than *Id3*$^{+/+}$ *Apoe*$^{-/-}$ mice (Figure 1). While it is possible that, in addition to B cells, other cell types could contribute to the increased atherosclerosis observed in the *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mouse, the use of the μMT *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 μMT *Apoe*$^{-/-}$ model Id3 is present in all cell types. Adoptive transfer of splenic B cells from *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice led to equal reconstitution of peripheral lymph tissue (spleen, lymph node and blood) as B cells from *Id3*$^{+/+}$ *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 (*Apoe*$^{-/-}$) appears to be important as neither 5[28] or 60[30] million B cells from B6 mice were atheroprotective when transferred into μMT *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 *Apoe*$^{-/-}$ mice null for CCR6 but wildtype 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[31] 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[32]. CCR6 is expressed on various cell types involved in atherosclerosis[33], and mice globally deficient in CCR6 have reduced circulating monocytes due to an reduced ability of these cells to leave the bone marrow[32], 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[34] suggesting that CCR6 may be a common chemokine receptor mediating B cell homing to tissue sites to modulate local inflammation[5, 6]. 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 *Id3*$^{-/-}$*Apoe*$^{-/-}$ mice is greater than that seen in *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[14, 24], however our results suggest that the ‘address’ for B cell homing to the aorta is independent of these factors. Grabner, 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 *Apoe*$^{-/-}$ mice[5]. Yet, in our studies of younger mice, B cells from *Id3*$^{+/+}$ *Apoe*$^{-/-}$ and *Id3*$^{-/-}$ *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 prior to 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[35]. As such, B cells from *Id3* null 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[26, 36–39] while IgG specific for these epitopes may be atherogenic[38, 40]. 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 *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice compared with control. Consistent with previous literature[18] examining antibody levels in young *Id3*$^{-/-}$ mice, the loss of Id3 in *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 *Id3*$^{-/-}$*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[5–14], 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, g, and h) 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 prior to feeding. Although μMT *Apoe*$^{-/-}$ mice do not have B cells in their aortas, they do have abundant macrophages. Reconstitution of the aorta of μMT *Apoe*$^{-/-}$ mice with *Id3*$^{+/+}$ *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 *Id3*$^{-/-}$ *Apoe*$^{-/-}$B cells were used (data not shown), suggesting that the effect is dependent on aortic B cell number. Notably, eight week old chow-fed *Id3*$^{-/-}$ *Apoe*$^{-/-}$ 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*$^{-/-}$ *Apoe*$^{-/-}$ and adoptively transferred μMT *Apoe*$^{-/-}$ mice cannot be directly compared due to the fact that 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,[5] suggesting that adventitial B

cells may pass signals into the vessel wall. Immunoglobulins are too large to pass through these conduits[5], 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*$^{-/-}$ *Apoe*$^{-/-}$ mouse provides a unique model with which to explore the cellular and molecular atheroprotective mechanisms of leukocytes resident in the aortic adventitia of non-diseased 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 (cIMT), 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-Standard Abbreviations and Acronyms

Id3	Inhibitor of Differentiation-3
ATLO	aortic tertiary lymphoid organs
HLH	helix-loop-helix
cIMT	carotid intima-media thickness
OxPL	Oxidized phospholipids
MDA	malondialdehyde
ApoB	apolipoprotein B
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester

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Novelty and Significance

What is known?

- B cells and immunoglobulins are in associated with atherosclerotic plaques in mice and humans, yet the factors regulating trafficking of B cells to the aorta and their impact on atherosclerosis are poorly understood.
- Inhibitor of Differentiation-3 (Id3) regulates gene expression in B cells and deletion of Id3 in *Apoe* $^{-/-}$ mice null increases atherosclerosis.
- Humans with a functionally significant polymorphism in the *Id3* gene have increased carotid intima-media thickness compared with those homozygous for the ancestral allele.

What new information does this article contribute?

- Id3 is necessary for early atheroprotection.
- Loss of Id3 reduces B cell CCR6 expression and deficiency of both ID3 and CCR6 reduces B cell aortic homing and B cell-mediated atheroprotection.
- B cells home to sites in the aorta prone to atherosclerosis and regions with early lipid deposition, reduce the macrophage content of the lesion and attenuate atherosclerosis progression.

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*$^{-/-}$ 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.

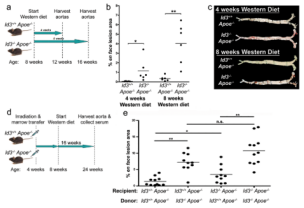

Figure 1. Loss of Id3 accelerates atherosclerosis development in *Apoe*$^{-/-}$ mice fed Western diet – an effect mediated by a bone marrow-derived cell type

a, Schematic of the experiment. Starting at 8 weeks of age, *Id3*$^{+/+}$ *Apoe*$^{-/-}$ or *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice were placed on a Western diet for 4 or 8 weeks duration. Aortas were perfused with paraformaldehyde, harvested, opened longitudinally and stained with Sudan IV. ***b,*** *En face* lesion area was quantitated using Image-Pro 5.0 software. *:p=0.004, **:p=0.002. ***c,*** Representative *en face* images are shown ***d,*** Schematic of the experiment. At 4 weeks of age, *Id3*$^{+/+}$ *Apoe*$^{-/-}$ or *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice were irradiated and transplanted with donor bone marrow, allowed to recover for four weeks, and then placed on a Western diet for 16 weeks. ***e,*** Aortas were then harvested and *en face* lesion area was quantitated as above. Each point represents a single animal. *:p=0.01, **:p=0.0005.

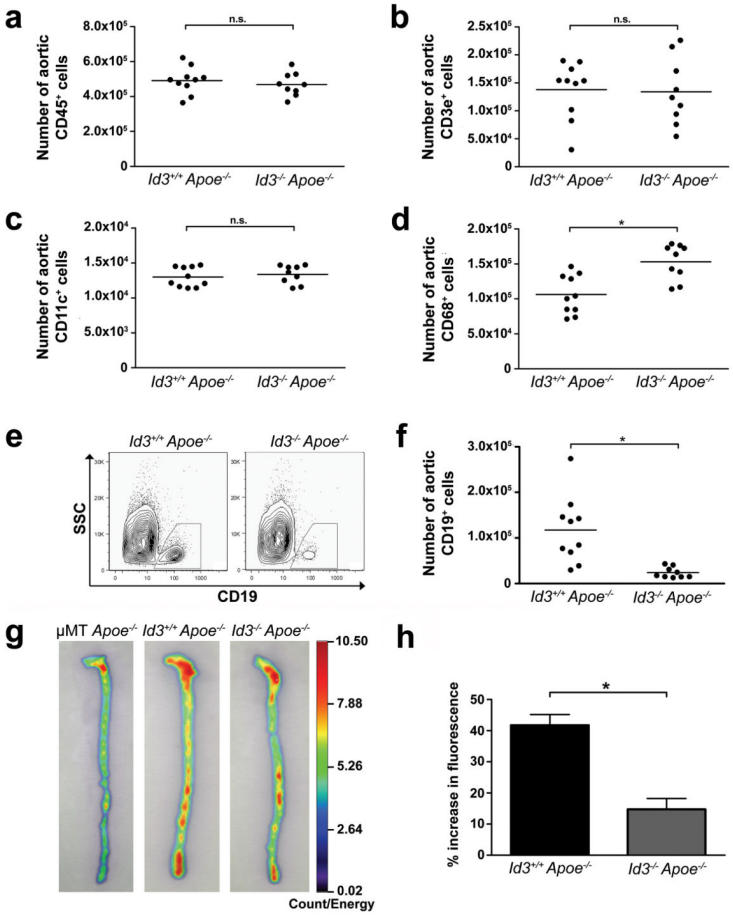

Figure 2. B cell number in the aortas of *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice is significantly decreased compared with the aortas of *Id3*$^{+/+}$ *Apoe*$^{-/-}$ mice

Aortas, spleens, lymph nodes and whole blood were harvested from chow-fed *Id3*$^{+/+}$ *Apoe*$^{-/-}$ or *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice at eight weeks of age. Each point represents an aorta from a single animal. Quantitation of aortic ***a,*** leukocytes (CD45^{+}). ***b,*** T cells (CD45^{+} CD3e^{+}). ***c,*** dendritic cells (CD45^{+} CD11c^{+}) and ***d,*** macrophages (CD45^{+} CD68^{+}). *p=0.003 ***e,*** Representative plots and ***f,*** quantitation of aortic B cells (CD45^{+} CD19^{+}). ***g,*** Representative 2-dimensional fluorescent reflectance images of aortas after incubation with Cy5.5-labeled anti-B220 antibody. ***h,*** Quantitation of mean fluorescence of *Id3*$^{+/+}$*Apoe*$^{-/-}$ and *Id3*$^{-/-}$ *Apoe*$^{-/-}$ mice. Data are represented as increase above mean fluorescence in the μMT *Apoe*$^{-/-}$ control group. Error bars reflect the SEM. *: p<0.0006.

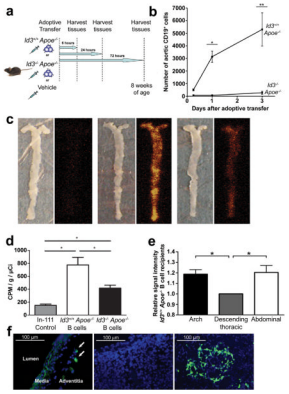

Figure 3. Id3 is necessary for B cell homing to the aorta

a, Schematic of the experiment. ***b,*** Quantitation of aortic B cells after adoptive transfer. Plotted values indicate the average B cell number obtained from six animals ± SEM for each time point. *:p=0.03, **:p=0.002. ***c,*** Representative *en face* images of aortas and corresponding ex vivo phosphor images of chow-fed μMT $Apoe^{-/-}$ recipient mice 20 hours following tail vein injection of In-111 control or 1×10^7 In-111 radiolabeled B cells from $Id3^{+/+}$ $Apoe^{-/-}$,or $Id3^{-/-}$ $Apoe^{-/-}$ mice. ***d,*** Quantitation of aortic radioactivity by gamma well counting (counts per minute/grams of aortic tissue/injected radioactive dose in μCi) Error bars represent SEM. *:p<0.05. ***e,*** Regional signal intensity analysis comparing the aortic arch and the abdominal aorta relative to the descending thoracic aorta for ex vivo phosphor images of $Id3^{+/+}$ $Apoe^{-/-}$ B cell recipient aortas. Error bar represents SEM. *:p<0.05. ***f,*** Left panel: Representative immunofluorescent images showing the location of CFDA-SE labeled $Id3^{+/+}$ $Apoe^{-/-}$ B cells within the adventitia 72 hours after adoptive transfer to chow-fed μMT $Apoe^{-/-}$ mice. Center panel: spleen negative control from μMT $Apoe^{-/-}$ mice that received PBS vehicle. Right panel: spleen positive control, demonstrating CFDA-SE labeled B cells

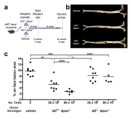

Figure 4. *Id3*$^{+/+}$ *Apoe*$^{-/-}$ but not *Id3*$^{-/-}$ *Apoe*$^{-/-}$ B cells confer atheroprotection to B cell-deficient μMT ApoE$^{-/-}$ mice

a, Schematic of the experiment. At eight 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,*** Quantitation of aortic lesion size. Each point represents a single animal. *:p<0.05, **:p<0.002, ***:p<0.001, ****:p<0.005.

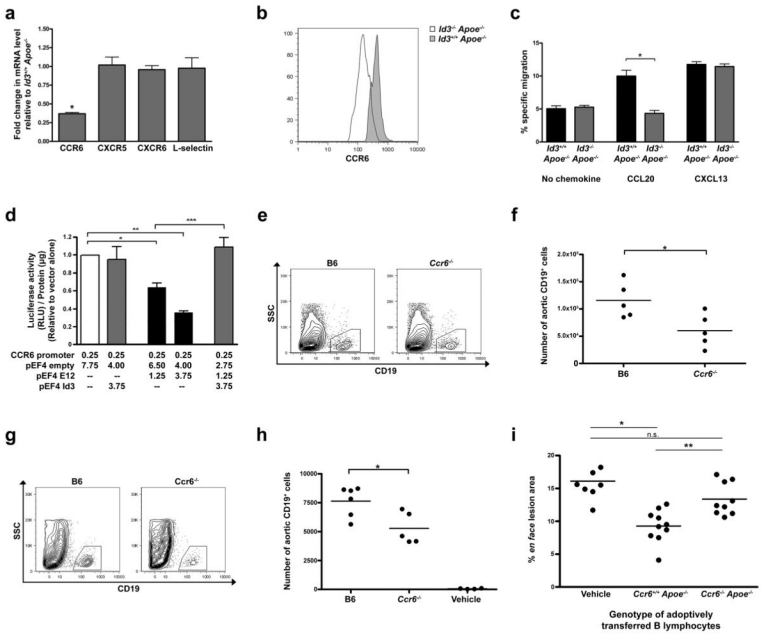

Figure 5. *Id3*$^{-/-}$ *Apoe*$^{-/-}$ B cells have decreased expression of CCR6 and expression of CCR6 on B cells mediates homing to the aorta and atheroprotection

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 co-transfected 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,*** quantitation 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,*** quantitation of B cell staining within aortas of μMT *Apoe*$^{-/-}$ mice 24 hours after adoptive transfer of 60 × 10^6 splenic B cells from B6 or *Ccr6*$^{-/-}$ mice or vehicle control. Each point represents a single animal. *p=0.009. ***i,*** Quantitation of *en face* lesion area of μMT *Apoe*$^{-/-}$ mice after adoptive transfer of 60 × 10^6 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, n.s. = not significant.

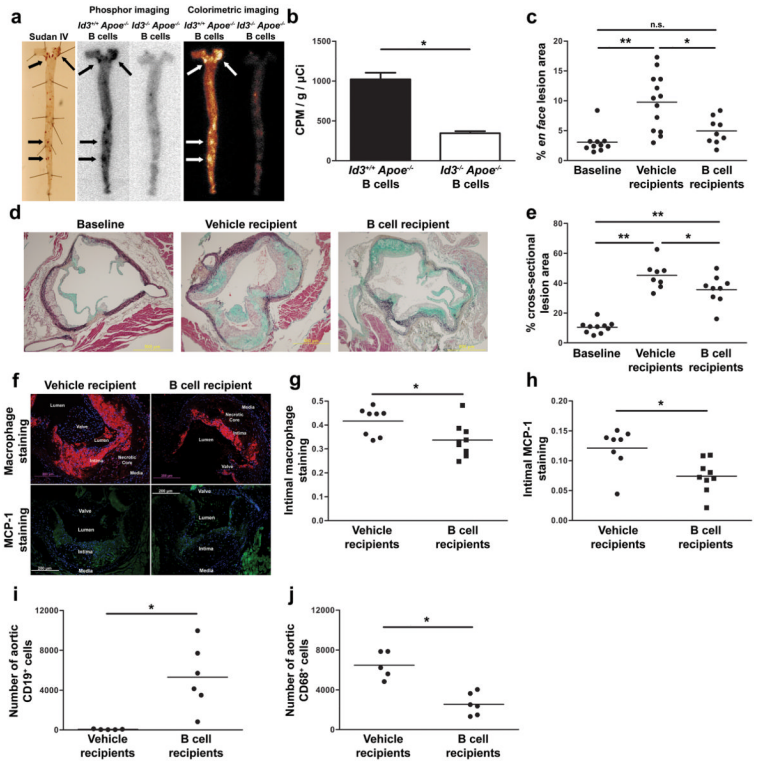

Figure 6. Adoptively transferred B cells traffic to regions of atherosclerosis, decrease plaque macrophage content and attenuate atherosclerosis progression

a, Sudan IV staining and *ex vivo* phosphor imaging of aorta from μMT *Apoe*$^{-/-}$ mice fed eight weeks of Western diet and injected with 2 × 10^{7} In-111 radiolabeled *Apoe*$^{-/-}$ or *Id3*$^{-/-}$*Apoe*$^{-/-}$B cells. ***b,*** Quantification (n=4) of counts adjusted for injected radioactivity and aortic weight. *:p=0.0003. ***c,*** Quantification of *en face* lesion area of μMT *Apoe*$^{-/-}$ mice fed 8 weeks of a Western diet (baseline, n=10) or injected with vehicle control (n=13) or 45 × 10^{6} *Apoe*$^{-/-}$ B cells (n=9) followed by an additional 8 weeks of Western diet *:p=0.011, **:p=0.0005, n.s. = not significant. ***d,*** Representative images and ***e,*** quantitation of MOVAT stained cross-sections at the aortic cusp *:p<0.05, **:p<0.001. ***f,*** Representative images and quantitation of ***g,*** macrophage and ***h,*** MCP-1 content in μMT *Apoe*$^{-/-}$ mice with 8 weeks of prior Western diet feeding that received injection of vehicle control or *Apoe*$^{-/-}$ B cells and an additional 8 weeks of Western diet feeding. Aortic cross sections (4 per animal at 60 μm intervals) were stained using DAPI (blue) and either a Mac-2 monoclonal antibody (red) or a MCP-1 polyclonal antibody (green) *:p=0.024 and 0.007 respectively. Quantitation of aortic ***i,*** B cell (CD19^{+} CD45^{+}) and ***j,*** macrophage (CD68^{+} CD45^{+}) content determined by flow cytometry 72 hours following adoptive transfer of either PBS vehicle injection or 60 × 10^{6} *Id3*$^{+/+}$ *Apoe*$^{-/-}$ B cells to chow-fed μMT *Apoe*$^{-/-}$ mice. *:p=0.006 and 0.0005 respectively.

Table 1

Number of B cells in the spleen, peri-aortic lymph nodes and whole blood.

	Id3$^{+/+}$ *Apoe*$^{-/-}$	*Id3*$^{-/-}$ *Apoe*$^{-/-}$	p-value
Peripheral B lymphocytes (×10^6 CD19$^+$ CD45$^+$ cells)			
Spleen	56.5 ± 10.4	56.3 ± 9.31	n.s.
Lymph nodes	0.89 ± 0.11	0.88 ± 0.14	n.s.
Blood (per ml)	1.96 ± 0.21	1.91 ± 0.29	n.s.

Values are the mean ± standard deviation. n.s.: not significant.

Table 2

Number of B cells in the spleen, peri-aortic lymph nodes and whole blood after adoptive transfer to μMT *Apoe*$^{-/-}$ mice

	Vehicle	*Id3*$^{+/+}$ *Apoe*$^{-/-}$	*Id3*$^{-/-}$ *Apoe*$^{-/-}$	p-value
Peripheral B lymphocytes (×10^5 CD19$^+$ CD45$^+$ cells)				
6 hours post-transfer				
Spleen		39.9 ± 0.59	37.6 ± 0.22	n.s.
Lymph node		1.37 ± 0.64	1.02 ± 0.41	n.s
Blood (per ml)		1.50 ± 0.40	1.60 ± 0.30	n.s
24 hours post-transfer				
Spleen	0.006 ± 0.001	72.1 ± 1.42	76.7 ± 1.37	n.s.
Lymph node	0.005 ± 0.001	1.11 ± 0.12	1.22 ± 0.19	n.s.
Blood (per ml)	0.006 ± 0.001	1.20 ± 0.30	1.00 ± 0.20	n.s.
72 hours post-transfer				
Spleen		94.5 ± 0.50	93.4 ± 0.58	n.s.
Lymph node		1.01 ± 0.10	1.12 ± 0.08	n.s.
Blood (per ml)		1.40 ± 0.70	1.50 ± 0.40	n.s.

Values are the mean ± standard error of the mean. n.s.: not significant.

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

	Id3$^{+/+}$ *Apoe*$^{-/-}$	*Id3*$^{-/-}$ *Apoe*$^{-/-}$	p-value
Peripheral B lymphocytes (×10^6 CD19$^+$ CD45$^+$ cells)			
30 × 10^6 B cells transferred			
Spleen	11.1 ± 1.31	10.6 ± 1.23	n.s.
Lymph nodes	1.86 ± 0.12	1.75 ± 0.17	n.s.
Blood (per ml)	0.29 ± 0.10	0.20 ± 0.10	n.s.
60 × 10^6 B cells transferred			
Spleen	16.9 ± 1.15	16.3 ± 0.81	n.s.
Lymph nodes	2.70 ± 0.16	2.60 ± 0.10	n.s.
Blood (per ml)	0.44 ± 0.20	0.29 ± 0.10	n.s.

Values are the mean ± standard error of the mean. n.s.: not significant.