Regulatory B Cell–Specific Interleukin-10 Is Dispensable for Atherosclerosis Development in Mice

Andrew P. Sage, Meritxell Nus, Lauren L. Baker, Alison J. Finigan, Leanne M. Masters, Ziad Mallat

Objective—To determine the role of regulatory B cell–derived interleukin (IL)-10 in atherosclerosis.

Approach and Results—We created chimeric Ldlr−/− mice with a B cell–specific deficiency in IL-10, and confirmed that purified B cells stimulated with lipopolysaccharide failed to produce IL-10 compared with control Ldlr−/− chimeras. Mice lacking B-cell IL-10 demonstrated enhanced splenic B-cell numbers but no major differences in B-cell subsets, T cell or monocyte distribution, and unchanged body weights or serum cholesterol levels compared with control mice. After 8 weeks on high-fat diet, there were no differences in aortic root or aortic arch atherosclerosis. In addition to plaque size, plaque composition (macrophages, T cells, smooth muscle cells, and collagen) was similar between groups.

Conclusions—In contrast to its prominent regulatory role in many immune-mediated diseases and its proposed modulatory role in atherosclerosis, B cell–derived IL-10 does not alter atherosclerosis in mice. (Arterioscler Thromb Vasc Biol. 2015;35:1770-1773. DOI: 10.1161/ATVBAHA.115.305568.)

Key Words: atherosclerosis ■ interleukins ■ lymphocytes

Atherosclerotic inflammation induces the recruitment and activation of adaptive (T and B cells) immunity. In humans, this is linked to clinically deleterious states of cardiovascular disease, for example unstable plaque. A key process may be the loss of tolerance to self antigens and progressive development of proinflammatory adaptive responses, whereby innate antigen presenting cells and the cytokine and danger-associated molecular patterns milieu favors Th1-type CD4+ helper cells. Like the more extensively characterized regulatory T cells, regulatory B (Breg) cells dampen dendritic cell and T-cell activation. A major anti-inflammatory effector mechanism used by regulatory B cells is the secretion of interleukin (IL)-10. B10 cells are a functionally defined population and include all B cells with the capacity to produce IL-10. They are enriched within the CD14+CD5+ population but they are not restricted to it. B-cell IL-10 production has been shown to protect against autoimmune disease development, including models of diabetes mellitus and adipose inflammation.

B2 cell depletion is atheroprotective whereas complete B-cell deficiency, that is, both B1 and B2 cells, promotes atherosclerosis. B cells, therefore, have multiple and opposing influences on atherosclerotic plaque development. Immunoglobulin M production by B1 cells is a major protective mechanism, whereas promotion of effector T-cell responses may mediate the proatherogenic potential of B2 cells. B1 cells can also produce substantial levels of IL-10 and the presence of a Breg cell subset among the B2 cell population suggests a potential counter-regulatory role in atherosclerosis. A recent study showed that reduction of aortic B1a and Breg cells was associated with reduced T15 IgM antibody and IL-10 levels, and increased atherosclerosis. We and others have previously shown an antiatherogenic role for IL-10, primarily when expressed by regulatory T cells or macrophages. To date, there have been no reports on the role of IL-10 from B cells or the role of Breg cells in atherosclerosis. Here, we show that contrary to current belief, B cell–restricted deficiency in IL-10 does not modulate atherosclerotic plaque development or modulate plaque phenotype.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
We first investigated whether regulatory B cells with IL-10 producing capability were present in atherosclerotic mice. In Ldlr−/− mice after 6 weeks of chow- or high-fat diet, we found a significant population of IL-10+ B cells in the spleen by intracellular flow cytometry, with no differences between chow- and high-fat diet–fed animals (Figure 1A). This indicates that innate B cells maintain high production of IL-10 in the presence of an inflammatory atherosclerotic setting.

To determine the specific role of B cell–derived IL-10 in atherosclerosis, we used a previously characterized mixed bone marrow chimera approach. Male mice were irradiated and reconstituted with 80% B cell–deficient (μMT) bone marrow cells and either 20% wild-type (WT) mice (Ldlr/BWT)
or 20% IL-10−/− bone marrow (Ldlr/B10−/−; Methods section of this article). After 4 weeks recovery, mice were fed a high-fat diet for 8 weeks. Circulating metabolic parameters (total cholesterol, high-density lipoprotein-cholesterol, insulin, and glucose) and animal weights were similar between the 2 groups of mice (Figure I in the online-only Data Supplement and data not shown). As expected, there were no differences in the number of Breg (CD19+ CD1dhi CD5+; Figure IIA in the online-only Data Supplement for an example) in spleen or lymph nodes (Figure IB and IIB in the online-only Data Supplement). Similar results were found with alternative gating (CD23hi CD21hi CD24hi; data not shown). However, IL-10 production in response to lipopolysaccharide was reduced 20-fold (mRNA levels) and 70-fold (protein levels) in B cells purified from the spleens of Ldlr/B10−/− compared with Ldlr/BWT mice (Figure 1C and data not shown) without any difference in IgM production (Figure 1C). There were no differences in IL-10 production by T cells (Figure IIC in the online-only Data Supplement) but circulating levels of IL-10 in serum were significantly reduced in Ldlr/B10−/− compared with Ldlr/BWT mice (Figure IID in the online-only Data Supplement). There was a small increase in total splenic B cells (Figure 1D), most probably because of an expansion of the follicular subset (Figure 1E). There were no differences in circulating monocytes but we observed a significant decrease in blood neutrophil numbers (Figure IIE in the online-only Data Supplement).

In other disease contexts, Breg cells have been reported to be important regulators of autoimmune T-cell responses. However, in the context of atherosclerosis, we did not find any significant impact on dendritic cell or T-cell activation levels (Figure III in the online-only Data Supplement) or T-helper polarization (data not shown). Accordingly, we did not see any significant changes in atherosclerosis development in the aortic root (Figure 2A), or in the aortic arch (Figure 2B).

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Breg</td>
<td>regulatory B cells</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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**Figure 1.** A, Levels of interleukin (IL)-10 producing B cells (CD19+) in the spleens of Ldlr−/− mice fed chow- or high-fat diet (HFD) for 6 weeks, quantified by intracellular flow cytometry after 4 hours ex vivo lipopolysaccharide (LPS) stimulation (Methods section of this article). B–E, Analysis of B cells from Ldlr−/− chimeric mice with µMT/wild-type (WT, BWT) or µMT/IL-10−/− (B10−/−) bone marrow after 8 weeks high-fat diet. B, Regulatory B-cell levels (CD19+ CD1dhi CD5+) in spleens. C, IL-10 and IgM production by B cells purified from spleens treated with or without LPS (1 µg/mL). D, Spleen B-cell (B220+ IgM+) levels. E, Spleen B-cell subsets defined by gating on CD23 and CD21 expression: T1 (CD23+ CD21−), T2 (CD23− CD21+), marginal zone (CD23+ CD21+), and follicular (CD23+ CD21+). *P<0.05.
observed a 1.4-fold increase in relative mRNA levels of inflammatory markers Ccl2 and Tnf but not Vcam-1 or Il6 (Figure 2C), indicating some impact of B-cell IL-10 deficiency in this location. However, this change was balanced by a 1.6-fold increase in Il10, presumably from other cell sources, suggesting that the overall influence of local cytokines is not changed in the BIL10−/− group. Indeed, although IL-10 mRNA expression was increased in the spleens of high-fat diet compared with chow-fed mice, analysis of B cells purified by negative magnetic bead-mediated separation and the corresponding positive fraction of non-B cells (Methods section in the online-only Data Supplement) indicated that the increased IL-10 expression derives primarily from non-B cells (Figure IV in the online-only Data Supplement). To determine if plaque morphology was different, we analyzed proportions of plaque macrophages, T cells, smooth muscle cells, and collagen content, but found no significant differences between groups (Figure 2D). We conclude that in contrast to other cellular sources of IL-10, regulatory B cell–derived IL-10 does not affect atherosclerosis development in Ldlr−/− mice.

Discussion

The oxidative modification of low-density lipoprotein and the accumulation of necrotic cell debris expose common immunogenic epitopes recognized by both natural and adaptive (induced) antibodies. These antibodies are produced by active B-cell responses that also produce IL-10 secreting Breg cells. This study is the first to show that this regulatory B-cell IL-10 response does not modulate early atherosclerosis development, a period during which significant pathogenic and protective B-cell responses are induced and regulate plaque progression.

B cells modulate atherosclerosis through several modalities. This is reflected in the apparently paradoxical results of different B cell–modulating strategies leading to either enhanced or decreased atherosclerosis.10–15,21 A clear role for IgM primarily from B1a plasma cells found in the spleen and bone marrow is now recognized, a role for spleen resident granulocyte–macrophage colony-stimulating factor–producing innate activator B cells in enhancing dendritic cell activation has recently been described,22 and while not proven directly a role for adaptive IgG responses and direct regulation of T cells also likely contribute to B-cell regulation of atherosclerosis.23 In the case of suppressive B cells, some reports suggest that IgM production is essential,24,25 whereas others show reduced atherosclerosis through IgM-independent pathways.21 Most recently, Gjurich et al16 show that, among other defects, L-selectin−/− mice with enhanced atherosclerosis lack aortic migration of IgM-producing B1a cells and IL-10–producing Breg cells. Our results suggest that these proatherogenic effects are not mediated through IL-10–producing Bregs. However, we cannot rule out IL-10–independent effects of Breg cells, for

![Figure 2. Analysis of atherosclerotic plaques of Ldlr−/− chimeric mice with µMT/wild-type (WT; BWT) or µMT/interleukin (IL)-10−/− (BIL10−/−) bone marrow after 8 weeks high-fat diet. A, Aortic plaque area quantified on 10 cryosections (n=10/group). B, En-face lesion area in the aortic arches. C, Quantitative polymerase chain reaction analysis of descending aorta RNA levels. D, Macrophage (MOMA2−), T cell (CD3+), smooth muscle cell (α-SMA+) and collagen (picrosirius red) content of aortic root plaques. *P<0.05.](image-url)
example, IL-35. More work is needed to define the activation status and migratory potential of B10 cells in the context of atherosclerosis, to better understand their differential impact on disease development compared with other inflammatory conditions. The role of gut-derived B-cell responses in atherosclerosis has yet to be investigated. Interestingly, regulatory B cells are much less suppressive against arthritis when derived from specific pathogen-free housed mice compared with conventionally housed mice. This may be due, in part, to differences in gut microbiota. It will be interesting to see if B-cell IL-10 has any role in conventionally housed atherosclerotic mice.

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Disclosures

None.

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Significance

B-cell responses are now recognized to have both protective and pathogenic influences on atherosclerosis development. Given the importance of the regulatory arm of T-cell immunity, it is significant to find that in fact IL-10–dependent regulatory responses of B cells are not important in atherosclerosis. This gives further insight into the potential consequences of targeting B cells in human cardiovascular disease patients.
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MATERIAL AND METHODS

Mice

Ldlr\(^{-}\) and \(\mu\)MT mice were purchased originally from Jackson labs and were on a C57Bl/6 background. Il-10\(^{-}\) mice were a kind gift of Dr Mekhola Malik, Department of Surgery, University of Cambridge. Male 6-8 week old Ldlr\(^{-}\) mice were lethally irradiated (9.5 Gy) then injected i.v. with \(1\times10^7\) bone marrow cells from donor mice. After 4 weeks recovery, mice were fed a high fat diet (21% Fat, 0.15% Cholesterol, Special Diet Services) for 8 weeks.

Flow cytometry

Single cell suspensions of mouse tissues were stained with fluorophore-conjugated antibodies and analyzed using an LSRII Fortessa (BD) flow cytometer. Data was analysed using FlowJo software (Miltenyi). Regulatory B cells were gated as CD19\(^+\) CD1d\(^{hi}\) CD5\(^+\). Similar data was obtained gating on B220\(^+\) IgM\(^+\) CD23\(^+\) CD21\(^{hi}\) CD24\(^{hi}\) T2/MZ progenitor cells. B cell subsets were defined (after gating on B220\(^+\) IgM\(^+\)) as T1 (CD23\(^-\) CD21\(^{-}\)), T2/MZP (CD23\(^+\) CD21\(^{hi}\)), marginal zone (CD23\(^{lo}\) CD21\(^{hi}\)) and follicular (CD23\(^+\) CD21\(^{lo}\)). Dead cells were excluded based on FSc, SSC and positive staining for Live/Dead Aqua (Life Technologies). Where necessary, intracellular staining was achieved using buffer kits from eBioscience. Cytokine expression was assessed in freshly isolated cells treated with leukocyte activation cocktail (BD) for 4-6h and for Breg, LPS (1 \(\mu\)g/ml).

Serum parameters

Serum lipids, glucose and insulin were quantified by the Core Biochemical Assay lab at the University of Cambridge. Serum II-10 was measured by ELISA (Peprotech).
B cell production of IL-10 and IgM

B cells were purified by negative magnetic selection and an AutoMACS Pro separator (Miltenyi). B cells (2 x 10^6) were incubated for 48h with or without LPS (1 µg/ml) in RPMI medium with 10% fetal calf serum, L-Glutamine, penicillin, streptomycin and 50 µM β-Mercaptoethanol. Levels of IL-10 and IgM in supernatants were quantified by ELISA using antibodies from Peprotech and Bethyl labs, respectively.

Analysis of atherosclerotic lesions

Cryosections (10 µm) of aortic root atherosclerotic lesions were stained with Oil Red O (plaque area), MOMA-2 (macrophages), CD3 (T cells), smooth muscle cells (α-sma) and collagen (picrosirius red). Images were captured and analysed using a Leica DM6000B microscope and accompanying software and analysed using ImageJ (NIH). Plaques in the aortic arch were analysed after Oil Red O staining using ImageJ and presented as % plaque area.

Quantitative PCR

RNA was extracted from descending aortas after homogenization in TRIzol (Life Technologies) using a tissue lyser (Qiagen) and a DirectZOL RNA extraction kit (Zymo). RNA was converted to cDNA using a Quantitect RT kit (Qiagen) and analysed using SYBR Green qPCR mix (Eurogentec) on a Roche Lightcycler. Results were normalized to reference genes hprt or rplp0 (36B4) (for purified B cells). Primer sequences are as follows (5’ to 3’): il10 (F GCTCTTACTGACTGGCATGAG and R CGCAGCTCTAGGACGATGTG), il6 (F GTTCTCTGGGAAATCGTGGA and R TGTACTCCAGGTAGCTATGG), tnf (F AGCCACGTCGTAGCAAACCAC and R
TCTATGGCCAGACCCTCACAC), *vcam1* (F ACGCTTGTTGAGCTCTGTGGG and R CCCAGGTGGAGGTCTACTCATTCCC) and *ccl2* (F TGTCATGCTTCTGGCCTGCTG and R GGGCGTTAACTGCTCTGGCTG).

**Statistics**

Results were analyzed in GraphPad Prism (LaJolla, CA, USA) using unpaired t-test, non-parametric Mann Whitney U Test (where values were not normally distributed) or two-way analysis of variance (aortic root atherosclerosis quantification) and presented as mean ± S.E.M. A P value of <0.05 was considered significant.
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SUPPLEMENTARY MATERIAL
**Supplemental Figure I**

**Figure I.** Serum total cholesterol (A), glucose (B), insulin (C) and high density lipoprotein cholesterol (D) levels in Ldlr<sup>-/-</sup> chimeric mice with µMT/WT (B<sup>WT</sup>) or µMT/IL-10<sup>-/-</sup> (B<sup>IL-10/-</sup>) bone marrow after 8 weeks high fat diet.
**Supplemental Figure II**

**A.** Representative gating of regulatory B cells as CD19\(^+\) CD1d\(^{hi}\) CD5\(^+\) cells. Analysis of lymph node regulatory B cells (B), spleen IL-10\(^+\) CD4\(^+\) T cells (C), serum IL-10 (D), and circulating monocytes and neutrophils (E) of Ldlr\(^{-/-}\) chimeric mice with µMT/WT (B\(^{WT}\)) or µMT/IL-10\(^{-/-}\) (B\(^{IL-10^{-/-}}\)) bone marrow after 8 weeks high fat diet. Monocytes were defined as CD11b\(^+\) CD115\(^+\) Ly6G\(^-\) and either Ly6C high (Hi), intermediate (Int) or low (Lo). Neutrophils (Neutro) were defined as CD11b\(^+\) Ly6G\(^+\) Ly6C\(^+\). *p<0.05.
Supplemental Figure III

A

CD40

MFI (cDCs)

B

CD80

MFI (cDCs)

C

MHCII

MFI (cDCs)

D

Effector CD4⁺ T cells

% CD4 T Cells

E

CD69⁺ CD4⁺ T cells

% CD4 T Cells

Figure III. Analysis of dendritic cell and T cell activation in the spleens of Ldlr⁻/⁻ chimeric mice with µMT/WT (B^WT) or µMT/IL-10⁻/⁻ (B^IL-10⁻/⁻) bone marrow after 8 weeks high fat diet. A-C. Dendritic cell expression of CD40 (A), CD80 (B) and MHCII (C). D and E. CD4⁺ T cell effector (CD62L⁻ CD44^hi) (D) and CD69⁺ CD4⁺ T Cells.
Supplemental Figure IV

Figure IV. Expression of IL-10 mRNA in whole spleen, spleen non-B cells or spleen B cells from chow or high fat diet-fed *ldlr*⁻/⁻ mice. *p<0.05.