# Regulatory B cell-specific interleukin-10 is dispensable for atherosclerosis development in mice

**Running Title:** B cell IL-10 and Atherosclerosis

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

*Division of Cardiovascular Medicine, Department of Medicine, University of Cambridge, Cambridge, UK, CB2 0SZ.*

Correspondence should be addressed to: Ziad Mallat, MD, PhD, at Division of Cardiovascular Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, CB2 2QQ, UK.

E-Mail: zm255@medschl.cam.ac.uk.

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**Abstract**

***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 LPS failed to produce IL-10 compared to 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 to 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.

Key words: atherosclerosis; lymphocytes; interleukins.

**Abbreviations:**

| | |
|---|---|
| Breg | Regulatory B cells |
| Il | Interleukin |
| Ig | Immunoglobulin |

## Introduction

Atherosclerotic inflammation induces the recruitment and activation of adaptive (T and B cell) immunity. In humans, this is linked to clinically deleterious states of cardiovascular disease, for example unstable plaque[1,2]. A key process may be the loss of tolerance to self antigens and progressive development of pro-inflammatory adaptive responses, whereby innate antigen presenting cells and the cytokine and danger-associated molecular patterns milieu favours Th1-type $CD4^+$ helper cells[3,4]. 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 utilized 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 $CD1d^{hi}$ $CD5^+$ population but they are not restricted to it[5]. B cell Il-10 production has been shown to protect against autoimmune disease development[6–8] including models of diabetes and adipose inflammation[9].

B2 cell depletion is atheroprotective whereas complete B cell deficiency, i.e. both B1 and B2 cells, promotes atherosclerosis[10–14]. B cells therefore have multiple and opposing influences on atherosclerotic plaque development[15]. Immunoglobulin (Ig)M production by B1 cells is a major protective mechanism, whereas promotion of effector T cell responses may mediate the pro-atherogenic potential of B2 cells. B1 cells can also produce substantial levels of Il-10[6,8] 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[16]. We and others have previously shown an anti-atherogenic role for Il-10[17,18], primarily when expressed by regulatory T cells[19] or macrophages[20]. 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 employed a previously characterized mixed bone marrow chimera approach[6,13]. *Ldlr*$^{-/-}$ male mice were irradiated and reconstituted with 80% B cell deficient (μMT) bone marrow cells and either 20% WT mice ('Ldlr/$B^{WT}$') or 20% *Il-10*$^{-/-}$ bone marrow ('Ldlr/$B^{Il10-/-}$') (see methods). After 4 weeks recovery, mice were fed a high fat diet for 8 weeks. Circulating metabolic parameters (total cholesterol, high density lipoprotein-cholesterol, insulin, glucose) and animal weights were similar between the two 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 ($CD19^{+}$ $CD1d^{hi}$ $CD5^{+}$; see Figure IIA in the online-only Data Supplement for an example) in spleen or lymph nodes (Figure 1B and IIB in the online-only Data Supplement). Similar results were found with alternative gating ($CD23^{hi}$ $CD21^{hi}$ $CD24^{hi}$; data not shown). However, Il-10 production in response to LPS was reduced 20-fold (mRNA levels) and 70-fold (protein levels) in B cells purified from the spleens of Ldlr/$B^{Il10\text{-}/\text{-}}$ compared to Ldlr/$B^{WT}$ 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/$B^{Il10\text{-}/\text{-}}$ compared to Ldlr/$B^{WT}$ mice (Figure IID in the online-only Data Supplement). There was a small increase in total splenic B cells (Figure 1D), most likely due to 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). In the descending aorta, we 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 $B^{IL10\text{-}/\text{-}}$ group. Indeed, although Il-10 mRNA expression was increased in the spleens of high fat diet compared to chow-fed mice, analysis of B cells purified by negative magnetic bead-mediated separation and the corresponding positive fraction of non-B cells (see methods 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). In order to determine if plaque morphology was different, we analysed 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 exposes common immunogenic epitopes recognised 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 recognised, 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 al[16] 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 pro-atherogenic effects are not mediated through Il-10 producing Bregs. However, we cannot rule out Il-10 independent effects of Breg cells, e.g. Il-35[26]. More work is needed to define the activation status and migratory potential of B10 cells in the context of atherosclerosis, in order to better understand their differential impact on disease development compared to 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 to conventionally housed mice[8]. 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**
The authors have no conflicts of interest to disclose.

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## Significance

B cell responses are now recognised 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.

## Figure Legends

**Figure 1**. **A**. Levels of Il-10 producing B cells ($CD19^+$) in the spleens of *Ldlr*$^{-/-}$ mice fed chow or high fat diet for 6 weeks, quantified by intracellular flow cytometry after 4h ex vivo LPS stimulation (see methods). ***B-E***. Analysis of B cells from *Ldlr*$^{-/-}$ chimeric mice with μMT/WT ($B^{WT}$) or μMT/IL-$10^{-/-}$ ($B^{IL\text{-}10\text{-}/\text{-}}$) bone marrow after 8 weeks high fat diet. **B**. Regulatory B cell levels ($CD19^+$ $CD1d^{hi}$ $CD5^+$) 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 ($CD23^+$ $CD21^{hi}$), marginal zone ($CD23^{lo}$ $CD21^{hi}$) and follicular ($CD23^+$ $CD21^{lo}$). *p<0.05.

**Figure 2**. Analysis of atherosclerotic plaques of *Ldlr*$^{-/-}$ chimeric mice with μMT/WT ($B^{WT}$) or μMT/IL-$10^{-/-}$ ($B^{IL\text{-}10\text{-}/\text{-}}$) 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 PCR 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.