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Conventional B2 B Cell Depletion Ameliorates whereas Its Adoptive Transfer Aggravates Atherosclerosis

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Atherosclerosis is a chronic inflammatory arterial disease characterized by focal accumulation of lipid and inflammatory cells. It is the number one cause of deaths in the Western world because of its complications of heart attacks and strokes. Statins are effective in only approximately one third of patients, underscoring the urgent need for additional therapies. B cells that accumulate in atherosclerotic lesions and the aortic adventitia of humans and mice are considered to protect against atherosclerosis development. Unexpectedly, we found that selective B cell depletion in apolipoprotein E-deficient (ApoE−/−) mice using a well-characterized mAb to mouse CD20 reduced atherosclerosis development and progression without affecting the hyperlipidemia imposed by a high-fat diet. Adoptive transfer of 5 × 10^6 or 5 × 10^7 conventional B2 B cells but not 5 × 10^6 B1 B cells to a lymphocyte-deficient ApoE−/− Rag-2−/− common cytokine receptor γ-chain–deficient mouse that was fed a high-fat diet augmented atherosclerosis by 72%. Transfer of 5 × 10^6 B2 B cells to an ApoE−/− mouse deficient only in B cells aggravated atherosclerosis by >300%. Our findings provide compelling evidence for the hitherto unrecognized proatherogenic role of conventional B2 cells. The data indicate that B2 cells can potentially promote atherosclerosis development entirely on their own in the total absence of all other lymphocyte populations. Additionally, these B2 cells can also significantly augment atherosclerosis development in the presence of T cells and all other lymphocyte populations. Our findings raise the prospect of B cell depletion as a therapeutic approach to inhibit atherosclerosis development and progression in humans. The Journal of Immunology, 2010, 185: 4410–4419.

Today, atherosclerosis is considered a chronic inflammatory disease of major arteries that leads to heart attacks and strokes caused by rupture of atherosclerotic plaques and thrombotic arterial occlusion. Current therapies for atherosclerosis are mainly based on drugs that lower plasma cholesterol concentration and blood pressure. The potent cholesterol-lowering agents (statins) significantly reduce cardiovascular events, not only as a consequence of their cholesterol lowering properties but also through their more recently described anti-inflammatory and immunomodulatory effects. However, atherosclerosis remains the main cause of heart attacks and strokes that are responsible for the majority of deaths in Western countries. The identification and development of promising new anti-inflammatory and immunomodulatory therapies is therefore of great interest in the management of atherosclerosis.

In early human plaques, B cells are minor populations found near lipid cores, whereas advanced human lesions contain adventitial nodular B cell aggregates (1, 2). In apolipoprotein E-deficient (ApoE−/−) mice, B cells are found in early fatty-streak lesions and accumulate in basal regions of advanced plaques containing high concentrations of proinflammatory cytokines TNF-α and IL-6 and abundant IgG and IgM deposits (3).

It is widely held that B cells protect against atherosclerosis development by producing IgM Abs to oxidized low-density lipoprotein (ox-LDL) (4, 5). This view is supported by the following observations: first, Ldlr−/− mice rendered B cell deficient by transplantation of bone marrow from μ-chain–deficient mice showed increased aortic atherosclerotic lesions accompanied by reduced anti–ox-LDL Abs (6). Secondly, Ldlr−/− mice, deficient in serum IgM, displayed substantially larger and more complex atherosclerotic lesions (7). The protective role of B cells in atherosclerosis has stimulated interest in developing a protective vaccine for atherosclerosis, targeted toward harnessing the protective B cell-derived IgM response to oxidized LDL (8).

B cell depletion mediated by mAb to CD20 remits a wide variety of inflammatory diseases in humans (9–12) and mice (13–16). In humans, rituximab, a chimeric Ab to human CD20, has been approved for treating intractable rheumatoid arthritis, although its value for other inflammatory diseases, such as systemic lupus erythematosus (10, 11) and multiple sclerosis (12), is currently being assessed. These observations highlight a key role for B lymphocytes in inflammatory diseases.

Based on the observations with B cell-depletion therapy, we revisited the role of B cells in atherosclerosis by depleting B lymphocytes using a mAb to mouse CD20 that has ameliorated murine thyroiditis (13), lupus (14), and arthritis (15). We found that B cell depletion prevented the development and progression of atherosclerosis. Moreover, transfer of conventional B2 B cells into a lymphocyte-deficient ApoE−/− Rag-2−/− common cytokine receptor γ-chain (γc)-deficient mouse or to B cell-deficient atherogenic mice lacking B lymphocytes due to deleted μ-chain of Ig...
 FIGURE 1. Experimental design and specific B cell depletion by anti-CD20 Ab. A–C. Schema of experimental design of B cell depletion and adoptive transfer of B2 B cells (n = 8–10/group). A. Depletion (prevention) study: ApoE<sup>−/−</sup> mice were fed a HFD for 4, 8, or 12 wk while maintaining B cell depletion by anti-CD20 Ab. B. Depletion (intervention study): ApoE<sup>−/−</sup> mice were fed a HFD for 10 wk during which two injections of anti-CD20 Ab were given. C. Adoptive transfer study: Ab administered. D. Single injection of anti-CD20 Ab depletes B cells up to 3 wk. ApoE<sup>−/−</sup> mice were given a single i.v. injection of anti-CD20 Ab and culled at days 3, 7, 14, and 21 postinjection. Shown are data for peripheral blood, spleen, and PC. Continuous and dotted lines, respectively, represent mice injected with anti-CD20 18B12 Ab (test) and 2B8 (control) Ab (n = 2 in each group). E. Repeated injections of CD20 Ab deplete >90% of B cells during atherosclerosis development. ApoE<sup>−/−</sup> mice were fed a HFD for 12 wk. Anti-CD20 Ab was given i.v. at the start of HFD and every 2 wk thereafter. Mice were culled after 4, 8, or 12 wk. Data show >90% depletion of CD22<sup>+</sup> B cells in mice fed a HFD for the 12 wk (n = 9 in each group). Similar B cell depletion efficiency was seen in both the 4 and 8 wk groups. F. Non-B lymphocytes and monocytes are unaffected by anti-CD20 Ab treatment. Shown are data for blood lymphocytes (CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-reg cells, NK1.1<sup>+</sup> NK cells, and NK1.1<sup>+</sup> TCR-β<sup>+</sup> NKT cells) and monocytes (CD11b<sup>+</sup> monocytes) in the 12 wk groups (n = 9 in each group). Similar findings were observed in spleen, PC, and lymph nodes in the 12-wk groups. G. Lipid profiles are unaffected by anti-CD20 Ab treatment. Shown are data from the depletion (prevention) 12-wk groups (n = 9 in each group). Similar data were seen in both depletion (prevention) 4- and 8-wk groups. White bars, mice injected with control (2B8) Ab. Black bars, mice injected with test (18B12) Ab. Data are mean values ± SEM. ***p < 0.01; ****p < 0.001. Blood, peripheral blood; LN, peripheral lymph nodes; PC, peritoneal cavity; T-reg, regulatory T cell.
potently aggravated atherosclerosis. In contrast, transfer of B1 B cells failed to aggravate atherosclerosis. Taken together, our studies clearly identify the B2 B cell subset as a proatherogenic B cell population.

Materials and Methods

Depleting Abs

A unique mouse anti-mouse CD20 IgG2a (18B12) used to deplete murine B cells and an isotype-matched control Ab (mouse anti-human CD20 Ab; 2B8) were supplied by Biogen Idec (San Diego, CA). The dosage used in depletion study was 10 mg/kg body weight for both Abs.

Animals and experimental design

In the depletion (prevention) study, 6–8-wk-old ApoE<sup>−/−</sup> mice were fed a high-fat diet (HFD; Specialty Feeds, Glen Forrest, Western Australia) for 4, 8, and 12 wk, during which either control Ab (2B8) or test Ab (18B12) was given once every 2 wk i.v. via tail vein (Fig. 1A). To induce atherosclerosis in the depletion (intervention) study, 6–8-wk-old ApoE<sup>−/−</sup> mice were fed an HFD for 6 wk and then given Ab treatment once every 2 wk for a further 4 wk on an HFD (Fig. 1B).

FIGURE 2. B cell depletion attenuates atherosclerosis development. A and B, Reduced atherosclerotic lesion development and macrophage accumulation. ApoE<sup>−/−</sup> mice were treated with anti-CD20 Ab fortnightly while being fed a HFD for 4, 8, or 12 wk (n = 9 in each group). Aortic sinus lesions were stained by Oil Red O (A, right panel) for lipid content or by anti-CD68 Ab (B, right panel) for macrophages. Arrows indicate positively stained areas. C and D, Reduction of lesion B cells. Shown are CD22<sup>+</sup> B cells (C) in aortic sinus lesions of mice treated with anti-CD20 Ab fortnightly while being fed a HFD for 12 wk compared with that in control group; lesion CD4<sup>+</sup> T cells (D) were unaffected (n = 9 in each group). Similar results were observed in the 8-wk group. Arrows indicate positively stained cells. E and F, Collagen and smooth muscle cell contents are unaffected by anti-CD20 Ab. Shown are collagen (E) and smooth muscle cell (F) contents in the 12-wk group (n = 9 in each group). Similar results were observed in all experiments of both prevention and intervention studies. Arrows indicate positively stained areas. Original magnification ×60 (A, B, E) and ×160 (C, D, F).

White bars, mice injected with control (2B8) Ab. Black bars, mice injected with test (18B12) Ab. Data are mean values ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
We generated a lymphocyte-deficient atherogenic ApoE<sup>−/−</sup> Rag-2<sup>−/−</sup> γc<sup>−/−</sup> (TKO) mouse model by crossing an ApoE<sup>−/−</sup> Rag-2<sup>−/−</sup> mouse and an ApoE<sup>−/−</sup> γc<sup>−/−</sup> mouse on a C57BL/6J background. We also generated an atherosclerosis-prone mouse deficient only in B cells by crossing an ApoE<sup>−/−</sup> mouse and a μMT<sup>−/−</sup> mouse lacking the μ-chain of Ig on C57BL/6J background. Genotype analysis confirmed the deleted gene status (data not shown). Phenotype analysis confirmed absence of all lymphocyte populations in the TKO mouse and absence of only B cells in the ApoE<sup>−/−</sup> μMT<sup>−/−</sup> mouse (data not shown).

In the transfer study (Fig. 1C), we adoptively transferred 5 × 10<sup>6</sup> or 5 × 10<sup>7</sup> spleen B2 B cells to 6–8-wk-old lymphocyte-deficient TKO mice at the start of an 8-wk HFD. The experiments were controlled by transfer of PBS and with the transfer of 5 × 10<sup>6</sup> peritoneal B1 B cells. In a separate study, we adoptively transferred 5 × 10<sup>4</sup> spleen B2 B cells to B cell-deficient ApoE<sup>−/−</sup> μMT<sup>−/−</sup> mice in a similar approach.

All animal experiments were conducted at Precinct Animal Centre, Alfred Medical Research and Education Precinct, Prahran, Victoria, Australia. All animal procedures were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee.

**Isolation of spleen conventional B2 B cells and peritoneal B1 B cells**

Conventional B2 B cells were purified from C57BL/6J spleens by using a B cell isolation kit containing a mixture of Abs against CD4, CD43, and Ter-113 (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s recommendation. Cell purity (>99%) was confirmed by FACS analysis using fluorochrome-labeled CD22 and CD5 Abs (BD Pharmingen, San Diego, CA). Cell viability (>98%) was assessed by trypan blue exclusion method (data not shown).

Peritoneal cells from C57BL/6J mice were obtained by flushing the peritoneum with FACS buffer and labeled with fluorochrome-conjugated CD22 and CD5 Abs (BD Pharmingen). To obtain 3 × 10<sup>6</sup> B1 B cells, a pool of peritoneal cells from 10 donor mice, each of which the peritoneum was flushed with 10 ml FACS buffer, was used. CD22<sup>+</sup> CD5<sup>+</sup> B1 B cells were isolated by FACS Aria flow sorter (BD Biosciences, San Jose, CA) to a purity of >99% (data not shown). Cell viability assessed by trypan blue exclusion method was >98% (data not shown).

**FACS analysis**

B cell and non-B cell populations in peripheral blood, peritoneal fluid, peripheral lymph nodes, and spleen were analyzed using fluorochrome-conjugated Abs (BD Pharmingen) on a BD FACS Canto II (BD Biosciences). For B cells and monocytes, PE-conjugated CD22, allophycocyanin-conjugated CD5, and allophycocyanin-Cy7-conjugated CD11b Abs were used. For non-B lymphocyte populations, Pacific Blue-conjugated CD4, PerCP-conjugated CD8α, allophycocyanin-Cy7-conjugated CD25, PE-conjugated Foxp3, allophycocyanin-conjugated TCR-β, and PE-Cy7-conjugated NK1.1 Abs were used. Data analysis was performed using BD FACSDiva software (BD Biosciences).

**Lipid analysis**

Plasma lipid profile was measured by a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyzer, with reagents and calibrators supplied by Beckman Coulter Diagnostics, New South Wales, Australia.

**Atherosclerotic lesion size analysis**

The heart and proximal aorta were dissected from mice, embedded in OCT compound (Tissue-tek, Sakura Finetek, Torrance, CA) and frozen at −80°C. Frozen sections (6 μm) were cut from the aortic sinus, defined as the region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off (17). The aortic sinus was evaluated because this region of the aorta is particularly susceptible to development of atherosclerosis in mice fed a HFD (17). Sections were stained with Oil Red O and examined using light microscopy and cross-sectional area of lipid deposition quantified using image analysis software (Optimas 6.2 Video Pro-32, Bedford Park, South Australia, Australia). For each mouse, lesion size was measured in 6 cross-sectional areas at 30 μm intervals and averaged. Collagen stained with Picosirius Red was analyzed using Polarizing microscope and Optimas software (Bedford Park).

**Immunohistochemical analysis**

Abs against CD68, CD22, CD4, and α smooth muscle actin were used in immunohistochemical analyses of macrophages, B cells, CD4<sup>+</sup> T cells, and...
smooth muscle cells in frozen sections of the aortic roots, respectively. CD68- and α-smooth muscle actin positively stained areas were quantified by Optimas software (Bedford Park). We microscopically counted CD22-stained B cells and CD4-stained T cells within atherosclerotic lesions in the intima of the aortic root. Mean values for positively stained areas and cell counts were calculated from three sections in the same way as described for the assessment of lesion size by Oil Red O stain.

**Measurement of ox-LDL–specific IgG and IgM Abs**

The ox-LDL–specific Abs were measured using ELISA. Briefly, copper-oxygenated human LDL and native human LDL (Calbiochem, Darmstadt, Germany) were used to coat 96-well ELISA plates at 50 µl of 10 µg/ml overnight at 4°C. Duplicate samples of 50 µl mouse plasma diluted 1:100 were added into the ELISA plates for 2 h at room temperature after blocking with 1% BSA, followed by addition of anti-mouse IgG or IgM Abs conjugated with HRP. Color development was done by addition of TMB solution, and plates were read at 450 nm wavelength. ox-LDL–specific Ab was determined by subtracting the native LDL OD from the ox-LDL OD.

**Measurement of plasma Igs**

To determine plasma Igs titers using ELISA, 50 µl anti-mouse Ig (1 µg/ml) was used to coat 96-well ELISA plates overnight at 4°C. After blocking with 1% BSA, duplicate samples of 50 µl plasma diluted 1:10^5 for IgM and 1:10^4 for IgM) was added into ELISA plates for 2 h at room temperature. Respective secondary anti-mouse Abs conjugated with HRP were added into the wells, followed by addition of TMB substrate for color development. The OD at 450 nm was read by ELISA reader.

**Analysis of gene expression**

Total RNA was extracted from aortic arches using TRIzol reagent (Invitrogen, Carlsbad, CA) and quantitated by measuring absorbance at 260 nm wavelength. cDNA was generated from 1 µg total RNA using TaqMan reverse transcription (Applied Biosystems, Foster City, CA) reagents according to the manufacturer’s recommendation. Quantitative gene expression analysis to determine the mRNA levels of TNF-α, IFN-γ, IL-2, IL-4, IL-12, and 18S was performed on an ABI PRISM 7500 real-time PCR system (Applied Biosystems) using SYBR Green technology (Applied Biosystems). Each reaction was analyzed in triplicate, and the changes in target gene expression levels were quantitated using the comparative cycle threshold method with 18S rRNA primers (Applied Biosystems) as an endogenous control (18, 19). Sequences of primers used were as follows: TNF-α forward, 5′-TCT TCT TGC TAC TGA ACT TCG-3′; TNF-α reverse, 5′-GAA GAT GAT CTT GGT GTG AGG-3′; IFN-γ forward, 5′-CTG GAC CTG TGG GTT GTG GAC-3′; IFN-γ reverse, 5′-CAA CAG CAA GGC GAA AAA GG-3′; IL-2 forward, 5′-CGC AGA GGT CCA AGT TCA TCT T-3′; IL-2 reverse, 5′-CAG GAT GCT CAC CTT CAA ATT TTA C-3′; IL-4 forward, 5′-CGA GCT CAC TCT CTG TGG TGT T-3′; IL-4 reverse, 5′-GGA GAT GGA TGT GCC AAA CG-3′; IL-12 forward, 5′-GGT GTA ACC AGA AAG GTG CG-3′; and IL-12 reverse, 5′-GAG GAA TTG TAA TAG CGA TCC TCC G-3′.

**Statistics**

GraphPad Prism 4 (GraphPad, San Diego, CA) was used for statistical analyses. Results are presented as mean ± SEM. Two-tailed unpaired Student t tests (for comparisons between two groups) or one-way ANOVA (for comparisons of ≥3 groups) were used for statistical analyses. The p values were considered significant at <0.05.

**Results**

**Mouse anti-mouse CD20 Ab (18B12) selectively depletes B cells in ApoE−/− mice**

To test the efficiency of anti-CD20–mediated B cell depletion in C57BL/6J ApoE−/− mice, we administered a single i.v. injection of mouse IgG2a anti-mouse CD20 Ab (18B12) and performed flow cytometric (FACS) analysis of B cells in peripheral blood and lymphoid tissues at days 3, 7, 14, and 21 postinjection. We observed sustained depletion of circulating B cells (>97%) through to the third week. Spleen and peritoneal B cells were depleted by >95 and >70%, respectively (Fig. 1D). Relative resistance of peritoneal B cells to anti-CD20–mediated depletion has been reported (20). We injected anti-CD20 Ab on each 2 wk to maintain B cell depletion.

To determine whether B cell depletion can prevent atherosclerosis development, we fed 6–8-wk-old C57BL/6J ApoE−/− mice.
an HFD for 4, 8, or 12 wk, during which we maintained B cell depletion by injecting anti-CD20 Ab (Fig. 1A). At the end of experiments, B cells remained depleted by 90–95% in peripheral blood, spleen, lymph nodes, and peritoneal cavity in anti-CD20–treated mice compared with control mice (Fig. 1E). Non-B lymphocytes and monocytes (Fig. 1F), total plasma cholesterol, LDL cholesterol, HDL cholesterol, triglyceride levels (Fig. 1G), and body weights (data not shown) were unaffected.

**B cell depletion by anti-CD20 Ab ameliorates atherosclerosis development in ApoE−/− mice**

To investigate the effect of B cell depletion on atherosclerosis development, we measured lipid content and macrophage accumulation within the intima of the aortic sinus. We observed marked reductions in B cell-depleted mice fed an HFD for 8 wk and 12 wk, but not for 4 wk. Lipid content (Fig. 2A) and macrophage accumulation (Fig. 2B) were reduced by 59 and 47%, respectively, in the 8-wk group and by 50% in the 12-wk group. CD22-stained B cells (Fig. 2C), but not CD4-stained T cells (Fig. 2D), were reduced in atherosclerotic lesions in aortic roots of anti-CD20–treated mice. Plaque stability assessed by collagen (Fig. 2E) and smooth muscle cell content (Fig. 2F) in relation to lesion size were unaffected in anti-CD20–treated mice.

**B cell depletion by anti-CD20 Ab retards established atherosclerosis in ApoE−/− mice**

Next, we asked whether CD20-targeted B cell depletion can reduce established atherosclerotic lesions. We employed an intervention approach in which 6–8-wk-old C57BL/6J ApoE−/− mice were initially fed an HFD for 6 wk to allow establishment of atherosclerosis before commencement of anti-CD20 Ab treatment (Fig. 1B). In this study, anti-CD20 Ab reduced B cells by 50% in the peripheral blood and 60% in the lymph nodes (Fig. 3A), whereas other lymphocyte and monocyte populations, body weight, and plasma lipids were unaffected (data not shown). Atherosclerotic lesion size was reduced by 46% (Fig. 3B) and macrophage accumulation by 33% (Fig. 3C) in the test group. Plaque stability assessed by smooth muscle and collagen contents was unaffected (data not shown). Lesion CD22+ B cell numbers and lesion CD4+ T cell numbers remained unchanged (data not shown), suggesting that activated B cells in established atherosclerosis may be more resistant to anti-CD20–mediated depletion.

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**FIGURE 5.** B2 B cell transfer increases atherosclerotic lesions in lymphocyte-deficient ApoE−/− mice. A and B, Dramatic increase in atherosclerotic lipid content and macrophage accumulation. Shown are aortic sinus lesions in TKO mice after spleen B2 B cell transfer (TKO-B2, black bars) compared with PBS transfer (TKO-PBS, white bars) (n = 8 in each group). Atherosclerotic lesions stained with Oil Red O (A) for lipid content and with anti-CD68 Ab (B) for macrophages. Arrows indicate positively stained areas. Original magnification ×60. C, Exaggerated atherosclerotic lesions in aortic arches. Shown are representative Oil Red O-stained aortic arches of TKO-B2 transfer group compared with TKO-PBS transfer group (n = 8 in each group). Arrows indicate Oil Red O-stained areas. Original magnification ×40. D, Lesion B cells identified in atherosclerotic lesions of TKO-B2 mice. Shown is aortic sinus lesion stained with anti-CD22 Ab for B cells in TKO-B2 transfer. Arrows indicate CD22+ B cells. Original magnification ×160. Data are mean values ± SEM. *p < 0.05. IA, innominate artery; LCCA, left common carotid artery; LS, left subclavian artery.
Transferred conventional B2 B lymphocytes are found in lymphocyte-deficient ApoE<sup>−/−</sup> mice

One potential mechanism by which anti-CD20 Ab attenuates atherosclerosis is by depleting conventional B2 B cells. To test this hypothesis, we reintroduced B2 B cells into a lymphocyte-deficient TKO mouse that is devoid of Rag-2–dependent lymphocytes (including B, T, and NKT cells) and γc-dependent NK cells. We used a B cell isolation kit (Miltenyi Biotec) to negatively select spleen B2 B cells from non-B cells and CD43-expressing B-1 B cells (21). Purity of the isolated B2 B cell preparation assessed by FACS analysis was >99% (Fig. 4A). We transferred 5 × 10<sup>6</sup> B2 B cells into lymphocyte-deficient TKO mice and fed the mice an HFD for 8 wk. At the end of the experiment, B2 B cells were present in peripheral blood, spleen, and peritoneal cavity, albeit in very small numbers of 0.1–0.3% compared with B cells in ApoE<sup>−/−</sup> mice (Fig. 4B).

Conventional B2 B lymphocytes potently augment atherosclerosis development

Despite the very small numbers of transferred B2 B cells in the peripheral blood, spleen, and peritoneal cavity of the lymphocyte-deficient TKO mice, atherosclerotic lesion size and macrophage accumulation in the aortic sinuses were dramatically increased by 72 and 53%, respectively (Fig. 5A, 5B), effects not observed following transfer of vehicle (PBS) only. Larger atherosclerotic lesions were also seen in the aortic arch (Fig. 5C). Transferred B2 B cells were present in these enlarged atherosclerotic lesions (Fig. 5D). Hyperlipidemia in the lymphocyte-deficient TKO mice was unaffected (data not shown).

In a separate experiment, we transferred 5 × 10<sup>7</sup> conventional B2 B cells into lymphocyte-deficient TKO mice. Atherosclerotic lesion size and macrophage accumulation were again increased, but they were not significantly different from those that followed 5 × 10<sup>6</sup> B2 B cell transfer (Fig. 6A). This result shows that 5 × 10<sup>6</sup> adoptively transferred B2 B cells is sufficient to accelerate atherosclerosis in lymphocyte-deficient TKO mice and supports the proatherogenic role of B2 B cells.

In another experiment, we examined the effect on atherosclerosis following the adoptive transfer of 5 × 10<sup>6</sup> conventional B2 B cells compared with the adoptive transfer of 5 × 10<sup>7</sup> B1 B cells into lymphocyte-deficient TKO mice. We found that only the transfer of B2 B cells, but not the transfer of B1 B cells, augmented atherosclerosis development (Fig. 6B).

Conventional B2 B lymphocytes also increase atherosclerosis development in ApoE<sup>−/−</sup> mice deficient only in B lymphocytes

Because TKO mice are deficient not only in B cells but also in non-B lymphocytes, we asked whether conventional B2 B cells are
levels of 3–16% of immunocompetent ApoE−/− mice selectively deficient only in B cells. We generated ApoE−/− μMT−/− mice deficient in the μ-chain of Ig and lacking only in B cells (data not shown). We then transferred 5 × 10⁶ conventional B2 B cells into these B cell-deficient ApoE−/− mice. Upon completion of 8 wk HFD, we found that the atherosclerotic lesions in ApoE−/− μMT−/− mice were increased >3-fold as compared with those from a control group that had not received B2 B cells (Fig. 6C).

Plasma Ig and ox-LDL–specific Ab levels

As conventional B cells are best known for Ab production, we examined plasma Igs as well as IgG and IgM Abs to ox-LDL. We used human LDL (Calbiochem) as a coating Ag in ELISA to compare the levels of ox-LDL–specific Abs in our studies because comparable ox-LDL–specific Abs against oxidized human LDL as coating Ag in ELISA have been reported in mice immunized with murine LDL (22). In our depletion (prevention) study, plasma Igs and ox-LDL–specific Abs remained unchanged with anti-CD20–mediated B cell depletion and 4 wk of HFD. With B cell depletion and 8 or 12 wk of HFD, total Igs and IgG but not IgM decreased (Fig. 7A), and only ox-LDL–specific IgG Ab decreased in the 12 wk group (Fig. 7B). In our depletion (intervention) study, total Ig, IgG, and IgM levels and IgG and IgM Abs to ox-LDL remained unchanged (Fig. 7C, 7D). The data suggest that atherosclerosis was ameliorated by B cell depletion irrespective of Ab levels. The observations are consistent with the resistance of long-lived plasma cells that do not express CD20 (23) to B cell depletion with anti-CD20 Ab (24). After B2 B cell transfer into TKO mice, Igs and ox-LDL–specific Abs absent in TKO mice, were detectable at low levels of 3–16% of immunocompetent ApoE−/− mice (Fig. 8A, 8B). Ig production by B cells in the absence of T cell help has been reported in T cell-deficient (25) and immunodeficient (26) mice.

TNF-α expression is increased in atherosclerotic lesions in TKO mice transferred with conventional B2 B lymphocytes

As B cells produce a broad range of pro- and anti-inflammatory cytokines (27, 28), we next examined atherosclerotic lesions, augmented by B2 B cell transfer into lymphocyte-deficient TKO mice, for cytokines by RT-PCR. We found elevated levels of TNF-α but not of IFN-γ, IL-2, IL-4, and IL-12 (Fig. 8C). To account for differences in lesion sizes, we expressed TNF-α levels relative to F4/80, a macrophage marker (Fig. 8D). TNF-α, as expressed as a proportion of macrophages, was increased but was not statistically significant (Fig. 8E).

Discussion

In the current study, we have shown that B cell depletion by anti-CD20 Ab not only prevents the development of atherosclerosis but also ameliorates established atherosclerosis. Contrary to the widely held view that B cells are protective (4, 5), our observations demonstrate that B cells can have a damaging role in atherosclerosis. We identified conventional B2 B cells as a proatherogenic B cell population because transfer of these B2 B cells into lymphocyte-depleted ApoE−/− mice or to B cell-deficient ApoE−/− mice potently augmented atherosclerosis development. These B2 B cells comprise the majority of circulating B cells and reside in lymphoid follicles in lymph nodes and in the spleen, where they are also known as follicular B cells. Augmented atherosclerosis development following transfer of B2 B cells into TKO mice totally lacking in Rag2-dependent T cells, B cells, NKT cells, and γc-dependent NK cells demonstrates that these conventional B2 B cells can directly promote atherosclerosis development entirely on their own in the complete absence of all other lymphocyte populations. Augmented atherosclerosis development following transfer of B2 B cells into ApoE−/− μMT−/− mice deficient only in B cells indicates that B2 B cells can also augment atherosclerosis development in the presence of all other lymphocyte populations. B2 B cells transfer into ApoE−/− deficient only in B cells

FIGURE 7. Igs and ox-LDL–specific Abs in B cell depletion studies. A and B, Igs and ox-LDL–specific Abs levels in 12-wk depletion (prevention) study. ApoE−/− mice were treated with anti-CD20 Ab and fed a HFD for 12 wk. A, Total Ig, IgG, and IgM. White bars, control mice injected with control (2B8) Ab; gray bars, test mice injected with test (18B12) Ab; open bar, total Ig; bar with vertical lines, IgG; bar with horizontal line, IgM. B, ox-LDL–specific IgG and IgM Abs. White bars, control mice injected with control (2B8) Ab; gray bars, test mice injected with test (18B12) Ab; bar with vertical lines, ox-LDL–specific IgG; bar with horizontal line, ox-LDL–specific IgM (n = 9 in each group). Data are mean values ± SEM. C and D, Igs and ox-LDL–specific Abs levels in depletion (intervention) study. ApoE−/− mice were fed a HFD for 6 wk, after which they were treated with anti-CD20 Ab while maintained on a HFD for a further 4 wk. C, Total Ig, IgG, and IgM; legend as in A. D, ox-LDL–specific IgG and IgM Abs; legend as in B (n = 9 in each group). Data are mean values ± SEM. *p < 0.05; **p < 0.01.
increased lesion size by >300%, whereas transfer into ApoE−/− mice deficient in all lymphocyte populations increased lesion size by 72%. The findings suggest that proatherogenic B2 B cells can act in concert with other proatherogenic lymphocyte populations to augment atherosclerosis development.

B cells are considered to have important roles in immunity by producing Abs. However, emerging evidence shows that B cells can influence immune responses without Ab involvement. For instance, in MRL/lpr mice, whereas B cell deficiency generated by Jh mutation prevented lupus nephritis (29), mice expressing a mutant transgene that prevented IgG secretion developed disease (30). With initial anti-CD20 treatment, plasma Igs were unaffected at 4 wk of HFD, but declined at 8 and 12 wk of HFD in subclass IgG but not IgM levels. These findings may reflect the relative resistance of IgM-producing B1 B cells to anti-CD20 Ab depletion (20). In contrast, plasma Igs and ox-LDL–specific Abs were largely unaffected by anti-CD20 treatment in established atherosclerosis. This may reflect the failure of anti-CD20 Ab treatment to deplete Ab-producing plasma cells (23, 24). The data suggest that atherosclerosis was ameliorated by B cell depletion irrespective of Ab levels. TKO mice did not show any detectable plasma Igs and ox-LDL–specific Abs, but those that received conventional B2 B cells were found to have very low plasma Igs and IgM Ab to ox-LDL compared with immunocompetent ApoE−/− mice. The presence of circulating Igs in the absence of T cell help is consistent with reports of Ig production by B cells from T cell-deficient (25) and immunodeficient mouse models (26). The significance, if any, for atherosclerosis of these low-level Abs generated without T cell help remains unknown.

In addition to Ab production, conventional B2 B cells produce a broad range of pro- and anti-inflammatory cytokines (27, 28). Significant increase in expression of TNF-α was detected in atherosclerotic lesions of TKO mice transferred with conventional B2 B cells. This finding was not significant when TNF-α expression levels were expressed relative to a macrophage marker, F4/80. Although a macrophage origin for TNF-α cannot be excluded, TNF-α is also produced by B lymphocytes (31, 32). It enhances B cell locomotion and migration (32) and is found in lesions in the vicinity of B lymphocytes (3). This finding suggests that B2 B cells may have augmented inflammation in a cytokine-dependent manner to promote atherosclerosis.

Although Ag presentation to proatherogenic CD4+ T cells by conventional B2 B cells can conceivably activate these T cells and further augment atherosclerosis, the notable finding in our study is that B2 B cells can promote atherosclerosis in the complete absence of T cells in the lymphocyte-deficient TKO mice. Our findings in these lymphocyte-deficient mice indicate that B cells can promote atherosclerosis in the absence of Ag presentation to

FIGURE 8. Abs and lesion TNF-α after B2 B cell transfer in TKO mice. A and B, Igs and ox-LDL–specific Abs levels. Data shown are Ab levels in TKO-PBS, TKO-B2, and ApoE−/− mice being fed a HFD for 8 wk. A, Open bar, total Ig; bar with vertical lines, IgG; bar with horizontal line, IgM. B, Bar with vertical lines, ox-LDL–specific IgG; bar with horizontal line, ox-LDL–specific IgM. C–E, TNF-α expression in atherosclerotic lesions of TKO-B2 and TKO-PBS mice. RT-PCR was carried out using cDNA derived from aortic arches as described in Materials and Methods. C, TNF-α expression. D, Macrophage marker F4/80 expression. E, TNF-α expression normalized by F4/80 expression. Data are mean values ± SEM. *p < 0.05; ***p < 0.001.
T cells. These findings are consistent with the suggestion that B cells may be important in the rate-limiting step in the genesis of autoimmune reactions (33). We suggest that our findings are likely to have relevance for other inflammatory diseases in which B cells have a role. Our identification of a proatherogenic role for B2 B cells provides a therapeutic target for attenuating lesion development/progression in hyperlipidemic subjects and other subjects susceptible to atherosclerosis-related cardiovascular complications.

Disclosures
The authors have no financial conflicts of interest.

References