Follicular B Cells Promote Atherosclerosis via T Cell–Mediated Differentiation Into Plasma Cells and Secreting Pathogenic Immunoglobulin G

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Objective—B cells promote or protect development of atherosclerosis. In this study, we examined the role of MHCII (major histocompatibility II), CD40 (cluster of differentiation 40), and Blimp-1 (B-lymphocyte–induced maturation protein) expression by follicular B (FO B) cells in development of atherosclerosis together with the effects of IgG purified from atherosclerotic mice.

Approach and Results—Using mixed chimeric *Ldlr*$^{-/-}$ mice whose B cells are deficient in MHCII or CD40, we demonstrate that these molecules are critical for the proatherogenic actions of FO B cells. During development of atherosclerosis, these deficiencies affected T–B cell interactions, germinal center B cells, plasma cells, and IgG. As FO B cells differentiating into plasma cells require Blimp-1, we also assessed its role in the development of atherosclerosis. Blimp-1-deficient B cells greatly attenuated atherosclerosis and immunoglobulin—including IgG production, preventing IgG accumulation in atherosclerotic lesions; Blimp-1 deletion also attenuated lesion proinflammatory cytokines, apoptotic cell numbers, and necrotic core. To determine the importance of IgG for atherosclerosis, we purified IgG from atherosclerotic mice. Their transfer but not IgG from nonatherosclerotic mice into *Ldlr*$^{-/-}$ mice whose B cells are Blimp-1-deficient increased atherosclerosis; transfer was associated with IgG accumulating in atherosclerotic lesions, increased lesion inflammatory cytokines, apoptotic cell numbers, and necrotic core size.

Conclusions—The mechanism by which FO B cells promote atherosclerosis is highly dependent on their expression of MHCII, CD40, and Blimp-1. FO B cell differentiation into IgG-producing plasma cells also is critical for their proatherogenic actions. Targeting B–T cell interactions and pathogenic IgG may provide novel therapeutic strategies to prevent atherosclerosis and its adverse cardiovascular complications.

Visual Overview—An online visual overview is available for this article. (***Arterioscler Thromb Vasc Biol*. 2018;38: e71-e84. DOI: 10.1161/ATVBAHA.117.310678.)**

Key Words: atherosclerosis ■ cell differentiation ■ cytokines ■ germinal center ■ immunoglobulin G

Atherosclerosis, a chronic inflammatory disease of large elastic and muscular arteries associated with lipid accumulation, is the underlying cause of heart attacks and strokes, the leading causes of global mortality.[1,2] The arterial inflammation is largely mediated by immune cells, including macrophages, T cells, and B cells.[3] Recent studies indicate a complex role for B cells in atherosclerosis,[4] whereby B1a and B1b cells protect against atherosclerosis[5,6] and B2 cells promote both development and progression of established atherosclerosis.[7] The mechanism by which B1a cells protect against atherosclerosis is by secreting protective natural IgM antibodies capable of neutralizing oxidized low-density lipoproteins and targeting leukocytes and T cells.[5,8] However, the mechanisms by which follicular B (FO B) cells promote atherosclerosis are not known.

Conventional B cells can in principle promote atherosclerosis by at least 3 different mechanisms: secreting proinflammatory cytokines; antigen presentation to T cells, including naïve T cells; and also differentiating into plasma cells producing immunoglobulins. Effector B2 B cells primed by Th1 cells and antigens secrete proinflammatory/proatherogenic cytokines, including TNF-α (tumor necrosis factor-α), IFN-γ (interferon-γ), and IL (interleukin)-12, while those primed by Th2 cells and antigens secrete IL-2 and IL-4,[9] cytokines implicated in atherosclerosis. B cell–derived TNF-α is a relatively minor contributor to atherosclerosis.[10] B2 cells also express major histocompatibility II (MHCII) and CD1d and, thus, can act as peptide and lipid antigen presenting cells affecting CD4 T cells and NKT (natural killer T) cells.[11,12] B cell–mediated

Received on: July 27, 2017; final version accepted on: March 14, 2018.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.117.310678/-/DC1.

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Nonstandard Abbreviations and Acronyms

Apoe$^{-/-}$	apolipoprotein E–deficient
Athero-IgG	IgG isolated from plasma of hyperlipidemic/atherosclerotic ApoE$^{-/-}$ mice
μMT	B cell–deficient
Blimp-1	B-lymphocyte-induced maturation protein
BM	bone marrow
Cd40$^{-/-}$	CD40-deficient
FO B	follicular B
HFD	high fat diet
hsp65	heat-shock protein 65
IFN-γ	interferon-γ
IL	interleukin
Ldlr$^{-/-}$	low-density lipoprotein receptor–deficient
MHCII	major histocompatibility II
***MhcII*$^{-/-}$**	MHCII-deficient
Tfh	follicular helper T
TNF-α	tumor necrosis factor-α
WT-IgG	IgG isolated from normolipidemic/nonatherosclerotic mice

antigen presentation contributes to pathogenic mechanisms implicated in acute allograft rejection[13]; antigen presentation by B cells also contributes to the pathogenesis of murine lupus. However, dendritic cells are recognized as potent initiators of CD4$^+$ T cell responses. Many humoral responses by B cells also involve antigen presentation. FO B cells activated by antigens via the B cell receptor present processed peptide antigens bound to MHCII to CD4$^+$ follicular helper T (Tfh) cells that recognize the antigen. Then, an interaction between CD40L (cluster of differentiation 40) on CD4$^+$ Tfh cells and CD40 on FO B cells promotes humoral responses by stimulating B cell proliferation and downstream responses, including humoral responses.[14,15]

Several lines of evidence implicate humoral B cell immune responses in the pathogenesis of atherosclerosis. Antibodies to hsp65 (heat-shock protein 65) have been associated with severe carotid atherosclerosis in humans.[16] Furthermore, autoantibodies against hsp65 promote atherosclerosis via mechanisms dependent on endothelial cell damage.[17] More recently, pathogenic roles for modified low-density lipoprotein antibodies in atherosclerosis have been suggested.[18] However, despite such studies, definitive evidence for FO B cell–dependent humoral mechanisms, including a role for IgG contributing to development of atherosclerosis, is lacking.[4]

Here using both loss and gain of function studies, we provide compelling evidence that FO B cells promote development of atherosclerosis via mechanisms dependent on their expression of MHCII and CD40. Mixed chimeric *Ldlr*$^{-/-}$ mice whose B cells were deficient in MHCII or CD40, molecules essential for the interaction of FO B cells with CD4$^+$ Tfh cells exhibited greatly reduced atherosclerotic lesions associated with large reductions in IgG. We used chimeric mice whose FO B cells are deficient in Blimp-1 (B-lymphocyte–induced maturation protein), a transcription factor required for FO B cell differentiation into plasma cells[19] to demonstrate that FO B cells promote atherosclerosis by differentiating to plasma cells. A critical role for IgG in promoting atherosclerosis was demonstrated by transferring IgG purified from atherosclerotic mice into chimeric *Ldlr*$^{-/-}$ mice whose FO B cells were deficient in Blimp-1.

Materials and Methods

Ethics and Animals

All animal experiments complied with national guidelines for care and use of laboratory animals and approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct, Melbourne, Australia. All mice were on a C57Bl6 genetic background. B cell–deficient (*μMT*$^{-/-}$) mice were maintained at Monash Medical Center Animal Facilities, Clayton, Australia. Wild-type (C57Bl6) mice, apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice, low-density lipoprotein receptor–deficient (*Ldlr*$^{-/-}$) mice, and CD40-deficient (*Cd40*$^{-/-}$) mice were maintained in the animal facility at Animal Ethics Committee of the Alfred Medical Research and Education Precinct. MHCII-deficient (*MhcII*$^{-/-}$; $A_\beta^{0/0}$) mice[20] were from the Melbourne University. *Blimp-1*$^{fl/fl}$*Cd23-Cre* mice were bred at the Walter and Eliza Hall Institute, Melbourne, and generated by crossing *Cd23-Cre* mice[21] with *Blimp-1*$^{fl/fl}$ mice.[22] We chose male mice in our study because virtually all previous studies on B cells and atherosclerosis used male mice; thus, we cannot exclude possible gender-dependent effects. All animal experiments were conducted at Precinct Animal Center, Animal Ethics Committee of the Alfred Medical Research and Education Precinct. Atherosclerosis was induced by feeding a high fat diet containing 21% fat and 0.15% cholesterol (HFD; Specialty Feeds, Glen Forrest, Western Australia) for 8 weeks. Sterile water was given ad libitum throughout the experiment.

Generation of Bone Marrow Chimeric *Ldlr*$^{-/-}$ Mice With B Cell Deficient in MHCII, CD40, and Blimp-1

Bone marrow (BM) transplantation using *Ldlr*$^{-/-}$ mice as recipients is a widely accepted and frequently used technology for altering specific genes in atherosclerotic mice[23,24] and in some instances exhibits advantages over cross-breeding.[25] We used a mixed chimera BM transplantation approach to delete CD40 and MHCII from B cells.[10,26] Briefly, 6-week-old male *Ldlr*$^{-/-}$ mice were subjected to whole-body irradiation with 10 Gy in 2 divided doses at 4 hourly intervals, followed by tail vein injection of 5 million BM cells comprising 80% from either μMT mice or wild-type mice and 20% from either *Cd40*$^{-/-}$ or *MHCII*$^{-/-}$ mice. In mice receiving BM from μMT mice all B cells are derived from either the *Cd40*$^{-/-}$ or *MHCII*$^{-/-}$ knockout BM and is sufficient to fully populate the B cell compartment of the recipient mice.[26] *Ldlr*$^{-/-}$ with B cells deficient in Blimp-1 were generated by transferring BM from *Blimp-1*$^{fl/fl}$*Cd23-Cre* mice into irradiated *Ldlr*$^{-/-}$ mice; control mice received BM from *Blimp-1*$^{+/+}$*Cd23-Cre* mice. Four weeks later when BM is fully reconstituted, the different chimeric mice were given ad libitum a HFD for 8 weeks.

Tissue Collection

Both experimental and donor mice were killed by using slow-fill carbon dioxide asphyxiation. Male donor mice were used to isolate BM cells from femur and tibia bones for transplantation into irradiated *Ldlr*$^{-/-}$ mice. After culling experimental mice blood was collected via cardiac puncture, the spleen for fluorescence-activated cell sorter (FACS) analyses, and hearts for assessment of atherosclerosis at the aortic root after embedding in optimal cutting temperature compound and freezing at −80°C. Blood was also collected for isolation and purification of IgG from atherosclerotic *Apoe*$^{-/-}$ (HFD for 23 weeks) and wild-type C57Bl6 mice.

IgG Purification

Plasma IgG purification was performed using affinity chromatography. Briefly, plasma samples from Chow-fed C57Bl/6 mice or HFD-*Apoe*$^{-/-}$ mice were pooled, diluted 4-fold in phosphate-buffered saline, filtered through 0.45 μmol/L filter, and loaded onto a HiTrap Protein G HP column (GE) using BioLogic DuoFlow Medium

Pressure Chromatography Systems (BioRad). After washing with phosphate-buffered saline, the IgG was eluted with glycine buffer (pH 2.8) and collected in neutralizing buffer (pH 8.5). The IgG was then dialyzed in 4 changes of phosphate-buffered saline overnight at 4°C. The amount of purified IgG was quantitated using Direct Detect method (Millipore) and purity assessed by SDS-PAGE (12%) electrophoresis under reducing and nonreducing conditions.

Plasma Lipids

Plasma concentrations of total cholesterol and very low–density lipoprotein/low-density lipoprotein cholesterol were determined enzymatically using a cholesterol assay kit (Roche/Hitachi) and automated chemistry analyzer.[7]

Flow Cytometry

Immune cells in peripheral blood and spleen were analyzed with fluorochrome conjugated antibodies (BD Pharmingen, San Diego, CA) on FACS-Canto II (BD Biosciences) as previously described.[7] Anti-CD19, anti-CD5, anti-IgM, anti-IgD, anti-CD23, anti-CD21, anti-CD1d, anti-MHCII, anti-CD11c, anti-CD11b, anti-CD44, anti-CD138, anti-IgD, anti-GL7, anti-CD4, anti-CD8, anti-TCR (T-cell receptor)-β, and anti-NK1.1 Abs were used in immune cell analysis. In intracellular staining, cells were stimulated for 5 to 6 hours with Cell Stimulation Cocktail plus Protein Transport Inhibitors (eBioscience, San Diego, CA). After blocking Fc receptors, surface markers were first stained with anti-TCR-β, anti-CD4, and anti-CD8 antibodies. Fixed and permeabilized cells were stained with anti-IFN-γ and anti-TNF-α antibodies. Data acquired on a FACS-Canto II (BD Biosciences) were analyzed using FACS-Diva software (BD Biosciences). Transitional T1 B cells were defined as $CD23^{-}IgM^{hi}IgD^{lo}CD21^{lo}CD19^{+}$, transitional T2 B cells as $CD23^{+}IgM^{hi}IgD^{hi}CD21^{lo}CD19^{+}$, marginal zone as $CD23^{-}IgM^{hi}IgD^{lo}CD1d^{+}CD21^{hi}CD19^{+}$, follicular as $CD23^{+}IgM^{hi}IgD^{hi}CD21^{int}CD19^{+}$, and B1a cells as $CD5^{+}CD1d^{+}CD23^{-}IgM^{hi}IgD^{lo}CD19^{+}$. $CD19^{-}$ splenocytes were used to analyze MHC^{+} and $CD40^{+}$ $CD11c^{+}$ dendritic cells.

Atherosclerosis Assessment

In accordance with the American Heart Association statement,[27] frozen sections (6 μm) were cut from optimal cutting temperature compound–embedded 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. For each mouse, intimal lesion areas were measured in 6 sequential cross-sectional areas at 80 μm intervals and averaged.[7] Total intimal lesion areas were measured in sections stained with hematoxylin and eosin; oil red-O–stained lipids within lesions were also quantified in a blinded manner using light microscopy imaging, Optimus 6.2 Video Pro-32 software, and a FVII Olympus camera.[7] For lesion necrotic core assessment, sections were stained with hematoxylin and eosin to identify acellular areas as necrotic cores and measured as described previously.[28] Apoptotic cells identified by terminal dUTP nick end-labeling under light microscopy were expressed per lesion areas as described before.[28]

Immunofluorescence

Frozen sections from atherosclerotic lesions were stained with appropriate nonimmune IgGs, anti-TNF-α, anti-IL-1β, anti-RANTES (regulated on activation, normal T-cell expressed and secreted), and anti-MCP-1 (monocyte chemotactic protein 1) antibodies as previously described.[28] Images were visualized under Olympus BX61 fluorescence microscope and images captured using FVII Olympus camera.

Enzyme-Linked Immunosorbent Assay

Plasma immunoglobulin (Ig) was determined using ELISA. Fifty microliter anti-mouse Ig (1 mg/mL) was used to coat 96-well ELISA plates overnight at 4°C. After blocking with 1% BSA, duplicate samples of 50 mL plasma (diluted $1:10^5$ for total Ig and IgG and $1:10^4$ for IgM) was added into ELISA plates for 2 hours 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.[7]

IL-1α and IL-6 ELISA kits (R&D systems) were used to determine the levels of these cytokines in affinity-purified plasma IgGs as per manufacturer's instructions.

Statistical Analysis

Statistical significance was assessed using a 2-tailed Student's *t* test or Mann–Whitney *U* test, depending on whether the data were normally distributed, as assessed by the Shapiro–Wilk test using GraphPad Prism program. Results were presented as mean±SEM. *P* values <0.05 were considered statistically significant.

Results

B Cell–Specific MHCII Expression Is Critical for the Proatherogenic Effects of FO B Cells

Tfh cell–dependent high affinity class switched antibody production is critically dependent on activated B cells presenting antigens to Tfh cells via MHCII.[29] To determine whether MHCII expression by FO B cells is critical for their proatherogenic effects, we generated mixed chimeric $Ldlr^{-/-}$ mice whose B cells were deficient in MHCII by transplanting BM from μMT chain–deficient mice or wild-type (WT) controls (80%) together with BM from mice deficient in MHCII (20%) into irradiated 6-week-old $Ldlr^{-/-}$ mice as previously described.[10,26] This approach creates a control mouse model with B cells, the majority of which are competent in MHCII, and a test mouse model with $MHCII^{-/-}$ B cells that allows comparable reconstitution of non-B immune cells, such as macrophages, monocytes, dendritic cells, T cells all of which are crucially important in atherosclerosis pathogenesis (Table I in the online-only Data Supplement). Additional chimeric mice that received mixed BM cells (80% μMT-chain deficient BM and 20% WT BM) were also generated to serve as an additional control mouse model for mice with B cells selectively deficient in either MHCII or CD40 molecule (Table I in the online-only Data Supplement). B cells in the chimeric mice do not express MHCII in contrast to control chimeric mice (Figure 1A), and atherosclerotic lesion size in $Ldlr^{-/-}$ mice whose B cells were deficient in MHCII was also reduced, by ≈45% compared with control mice ($P<0.05$; Figure 1B and 1C), while the content ratio of lipid in lesions was unaffected ($P>0.05$; Figure 1B and 1C). MHCII expression by dendritic cells and macrophages is unaffected (Figure 1D and 1E; Figure IA and IB in the online-only Data Supplement). In an agreement with the literature where deletion of MHCII in B cells has previously been shown not to affect B cell numbers, marginal zone, T-1 or T-2 immature B cells in spleen,[30] we found similar observation in our mixed chimeric mice (Figure IIA and IIB in the online-only Data Supplement). Also, MHCII deletion in B cells has no effect on splenic B, $CD8^{+}$ and $CD4^{+}$ T cells, NK and NKT cells, monocyte or macrophage numbers at the beginning (Figure IIC in the online-only Data Supplement) and at the end (data not shown) of HFD. MHCII deletion in B cells significantly reduced the number of activated $CD44^{hi}CD4^{+}$ T cells in spleens of $Ldlr^{-/-}$ mice but did not affect $CD8^{+}$ T cells ($P<0.05$; Figure 1F; Figure IIIA in the online-only Data

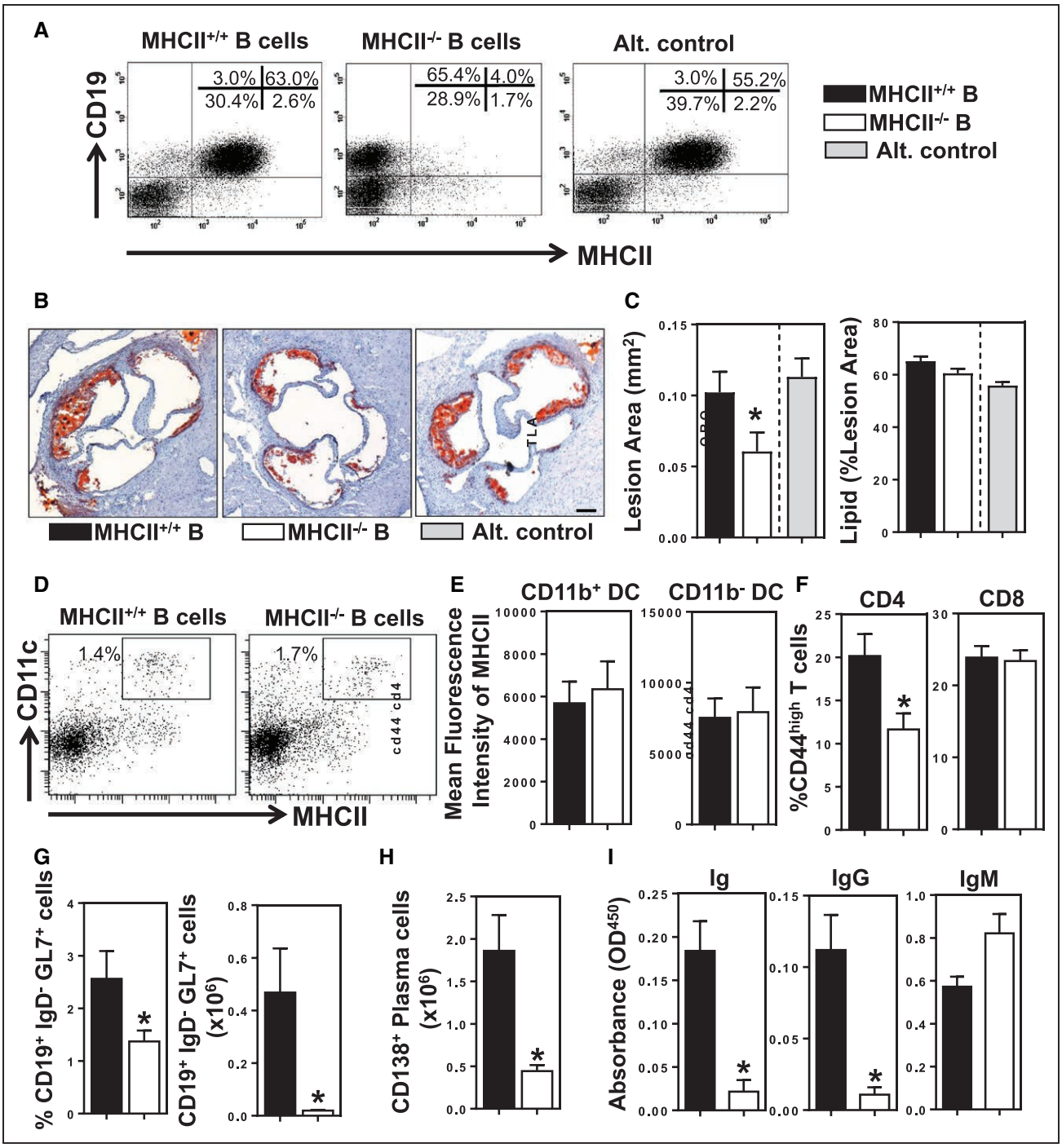

Figure 1. B cells require MHCII (major histocompatibility II) molecule to promote atherosclerosis. **A**, B cells do not express MHCII and (**B** and **C**) atherosclerosis reduces without affecting lesion lipid content ratio in mixed chimeric *Ldlr*$^{-/-}$ mice (MHCII$^{-/-}$ B cells; 80% μMT$^{-/-}$ plus 20% MHCII$^{-/-}$ bone marrow) compared with control mice (MHCII$^{+/+}$ B cells; 80% μMT$^{-/-}$ plus 20% wild-type (WT) bone marrow and Alt. control; 80% WT plus 20% MHCII$^{-/-}$ bone marrow). **D** and **E**, Without affecting MHCII expression on dendritic cells, chimeric *Ldlr*$^{-/-}$ mice with B cells deficient in MHCII reduce the numbers of spleen (**F**) CD4 T cells, (**G**) germinal center B cells, and (**H**) plasma cells. **I**, Plasma Ig and IgG, but not IgM, are greatly reduced by MHCII deficiency in B cells. *P<0.05; results are means±SEM, n=13 to 15 per group. MHCII$^{-/-}$ B; mice with B cells selectively deficient in MHCII, MHCII$^{+/+}$ B, and Alt. control; control mice. P<0.05 from control; results are means±SEM, n=13 to 15 per group. Scale Bar, 100 μm.

Supplement). Further analysis also indicated that B cell–specific MHCII deficiency reduced CD44+ PD-1^{+} Bcl6^{+} CD4 Tfh cells not only in CD4 T cell percentage but also in numbers by 52% and 68%, respectively (Figure IVA and IVB in the online-only Data Supplement). These effects were associated with reductions in the number of spleen CD4^{+} T cells expressing IFN-γ and TNF-α (P<0.05; Figure IIIB in the online-only Data Supplement) and cytokines that are proatherogenic and can also contribute to antibody production.[31,32] We used GL7 to assess effects on germinal center B

cells, a well accepted marker for germinal center B cells[33]; activated B cells do not express GL7.[34] MHCII deletion in B cells also greatly reduced CD19$^+$IgD$^-$GL7$^+$ germinal center B cells (P<0.05; Figure 1G), which was accompanied by a large (76%; P<0.05) reduction in splenic CD138$^+$ plasma cells (Figure 1H). These effects were associated with large (>90%; P<0.05) reductions in plasma total Ig and IgG levels without affecting IgM levels (Figure 1I). B cells deficiency in MHCII did not affect lesion macrophages, CD3 T cells, CD19 B cells, or lesion collagen (Table II in the online-only Data Supplement). Plasma cholesterol levels were also unaffected (P>0.05; Figure IIIC in the online-only Data Supplement).

Deletion of CD40 in FO B Cells Reduces Severity of Atherosclerosis and Is Associated With Reduced Plasma IgG

CD40 expression by FO B cells is essential for formation of germinal centers, B-cell proliferation within such centers, and immunoglobulin class switching from IgM to IgG.[35] CD40 is also required for upregulation of CD80/CD86 costimulatory molecules and subsequent T-cell stimulation.[36] As CD40 on FO B cells is activated by CD40L expressed by CD4$^+$ Tfh cells,[37] we examined the effects on atherosclerosis of deleting CD40 from FO B cells in *Ldlr*$^{-/-}$ mice. We generated mixed chimeric *Ldlr*$^{-/-}$ mice by transplanting BM from μMT chain–deficient mice or WT controls (80%) together with BM from mice deficient in CD40 (20%) into irradiated 6-week-old *Ldlr*$^{-/-}$ mice as previously described,[10] as well as additional chimeric mice that received mixed BM cells (80% μMT chain–deficient BM and 20% WT BM; Table I in the online-only Data Supplement). These chimeric mice contain B cells with greatly reduced CD40 (Figure 2A), and B cell–selective CD40 deficiency reduced atherosclerosis in aortic sinus compared with atherosclerotic lesions in the control, as well as in additional control mice (Figure 2B and 2C), and in the innominate artery (Figure VA in the online-only Data Supplement). CD40 expression by dendritic cells and macrophages is unaffected (Figure 2D and 2E; Figure IC and ID in the online-only Data Supplement). In these mice, major lymphocyte populations and plasma IgM were unaffected by reduction in CD40 on B cells (Figure VB and VC in the online-only Data Supplement). Assessment at the beginning of HFD also showed that mature B cells, immature T1 and T2 cells, and B1a cell numbers were normal in accordance with pervious report,[35] while marginal zone B cells were slightly elevated (Figure VD in the online-only Data Supplement) that became at a similar level to control mice at the completion of the experiment (Figure VE in the online-only Data Supplement). The large reduction in CD40 in B cells resulted in a 78% reduction in the number of CD19$^+$IgD$^-$GL7$^+$ germinal center B cells (P<0.05; Figure 2F) as well as a 65% reduction in spleen plasma cells (P<0.05; Figure 2G). Also activated spleen CD44^{hi}CD4$^+$ T cells and CD4$^+$IFN-γ$^+$ T cells were reduced by 27% and 46%, respectively (P<0.05), while activated CD8$^+$ T cells and CD4$^+$TNF-α$^+$ T cells are unaffected (Figure 2H; Figure VIA in the online-only Data Supplement). B cell–specific CD40 deficiency also reduced CD44$^+$ PD-1$^+$ Bcl6$^+$ CD4 Tfh cells not only in CD4 T cell percentage (by 48%) but also in numbers (by 58%), respectively (Figure IVA and IVC in the online-only Data Supplement). FO B cells deficient in CD40 also reduced plasma total Ig and IgG levels by 75% and 73%, respectively (P<0.05); however, IgM levels unaffected (Figure 2I). Plasma cholesterol levels were unaffected (P>0.05; Figure VIB in the online-only Data Supplement). B cells deficient in CD40 did not affect lesion macrophages, CD3 T cells, CD19 B cells, or lesion collagen (Table II in the online-only Data Supplement),

Total body irradiation affects neointimal smooth muscle cells in blood vessels, as occurs after arterial injury rather than medial smooth muscle cells[38]; neointimal smooth muscle which exhibit a synthetic phenotype and are highly proliferative differ from medial smooth muscle which exhibit a "contractile phenotype" and are quiescent.[39] To confirm that irradiation does not affect medial smooth muscle cells, the only smooth muscle cell type present in the aortic sinus of 6-week-old mice, we compared smooth muscle cells in medial layers in chimeric *Ldlr*$^{-/-}$ mice with B cells deficient in CD40 to HFD-fed ApoE and C57BL/6 mice that have not been exposed to total body irradiation. We did not see any effect of irradiation on vascular smooth muscles in both medial and intimal layers (Figure VII in the online-only Data Supplement).

FO B Cell Blimp-1 Deletion Prevents Development of Plasma Cells and Greatly Attenuates Atherosclerosis

Our studies suggest that IgGs produced by spleen plasma cells may be important in development of atherosclerosis. However, both MHCII and CD40 expression by B cells have the potential to stimulate naïve CD4$^+$ T cells to some extent in addition to promoting humoral adaptive immunity. To directly assess the importance of adaptive humoral immunity independently of naïve CD4$^+$ T cell stimulation, we generated chimeric mice whose B cells were deficient in Blimp-1 by transplanting BM from *Blimp-1*$^{fl/fl}$*Cd23-Cre* mice (100%) into irradiated *Ldlr*$^{-/-}$ mice. Blimp-1 is required for the formation of immunoglobulin-secreting plasma cells and their survival as well as preplasma memory B cells.[40] Mature B cells, immature T1 and T2 cells, marginal zone B cells, and B1a cells in spleens were unaffected by Blimp-1 deficiency in B cells (P>0.05; Figure 3A). Activated spleen CD44^{hi}CD4+ and CD8+ T cell numbers were also unaffected (P>0.05; Figure 3B). In contrast, plasma cell numbers were reduced by >90% (Figure 3C). Plasma total Ig and IgG levels were also reduced by >95%, while plasma IgM levels were reduced by nearly 80% (P<0.05; Figure 3D). Critically, IgG in atherosclerotic lesions was undetectable (Figure 3E), and aortic lesion size was reduced by nearly 50% (P<0.05; Figure 3F and 3G). B cells' deficiency in Blimp-1 did not affect lesion macrophages, CD3 T cells, CD19 B cells, or l collagen (Table II in the online-only Data Supplement). Neither were lesion lipid content ratio nor plasma lipids affected by Blimp-1 deletion from B cells (P>0.05; Figure 3F through 3H).

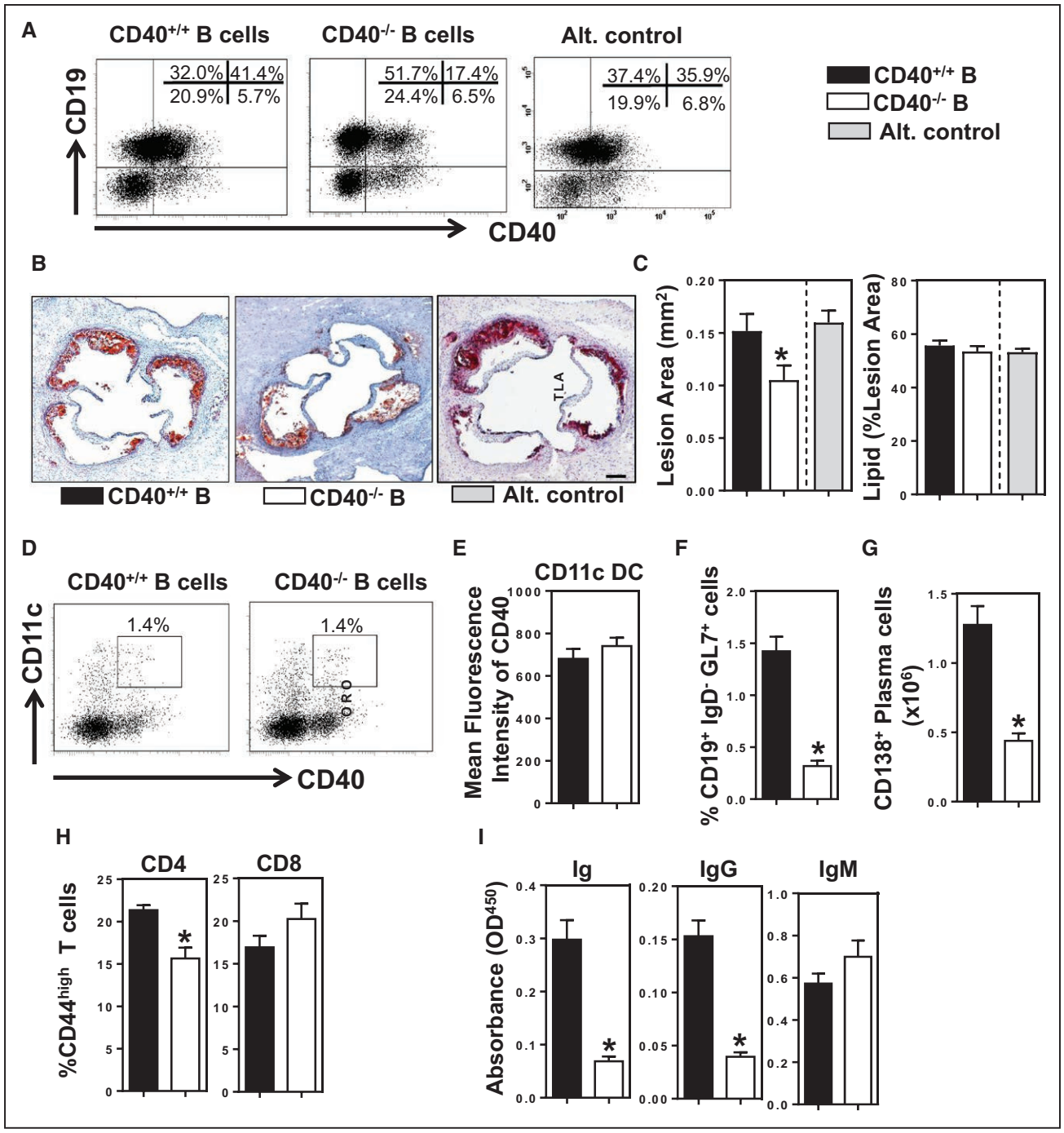

Figure 2. B cell–specific CD40 (cluster of differentiation 40) deficiency reduces atherosclerosis. **A**, B cells exhibit greatly reduced CD40 expression and (**B** and **C**) atherosclerosis reduces without affecting lesion lipid content in *Ldlr*$^{-/-}$ mixed chimeric mice (CD40$^{-/-}$ B cells; 80% μMT$^{-/-}$ and 20% CD40$^{-/-}$ bone marrow) compared with control mice (CD40$^{+/+}$ B cells; 80% μMT$^{-/-}$ and 20% WT bone marrow and Alt. Control; 80% WT$^{-/-}$ and 20% CD40$^{-/-}$ bone marrow). **D** and **E**, Without affecting dendritic cells, (**F**) germinal center B cells and (**G**) plasma cells are greatly reduced in hyperlipidemic *Ldlr*$^{-/-}$ mice with reduced CD40 express by B cells. **H**, Activated CD44^{hi} CD4+ cells but not CD8+ spleen T cells are reduced in mixed chimeric *Ldlr*$^{-/-}$ mice with reduced CD40 expression by B cells. **I**, Plasma Ig and IgG but not IgM are reduced in hypercholesterolemic mixed chimeric *Ldlr*$^{-/-}$ mice with B cells deficient in CD40. *P<0.05; results are means±SEM; n=9 to 11 per group. CD40$^{-/-}$ B; mice with B cells selectively deficient in CD40, CD40$^{+/+}$ B, and Alt. control; control mice. Scale Bar, 100 μm.

IgG From Hyperlipidemic/Atherosclerotic Mice Promote Development of Atherosclerosis

Our studies suggest a close relationship between the extent of atherosclerosis and plasma IgG. To directly test the hypothesis that IgG produced during development of atherosclerosis contributes to the proatherogenic effects of FO B cells, we examined the effects of IgG isolated from plasma of hyperlipidemic/atherosclerotic ApoE$^{-/-}$ mice (Athero-IgG) compared with IgG from normolipidemic/nonatherosclerotic mice (WT-IgG) on atherosclerosis development in *Ldlr*$^{-/-}$ mice whose B cells are deficient in Blimp-1. IgG of 10 μg/dose (purity >95%; Figure 4A) in which none of IL-1α and IL-6 proinflammatory

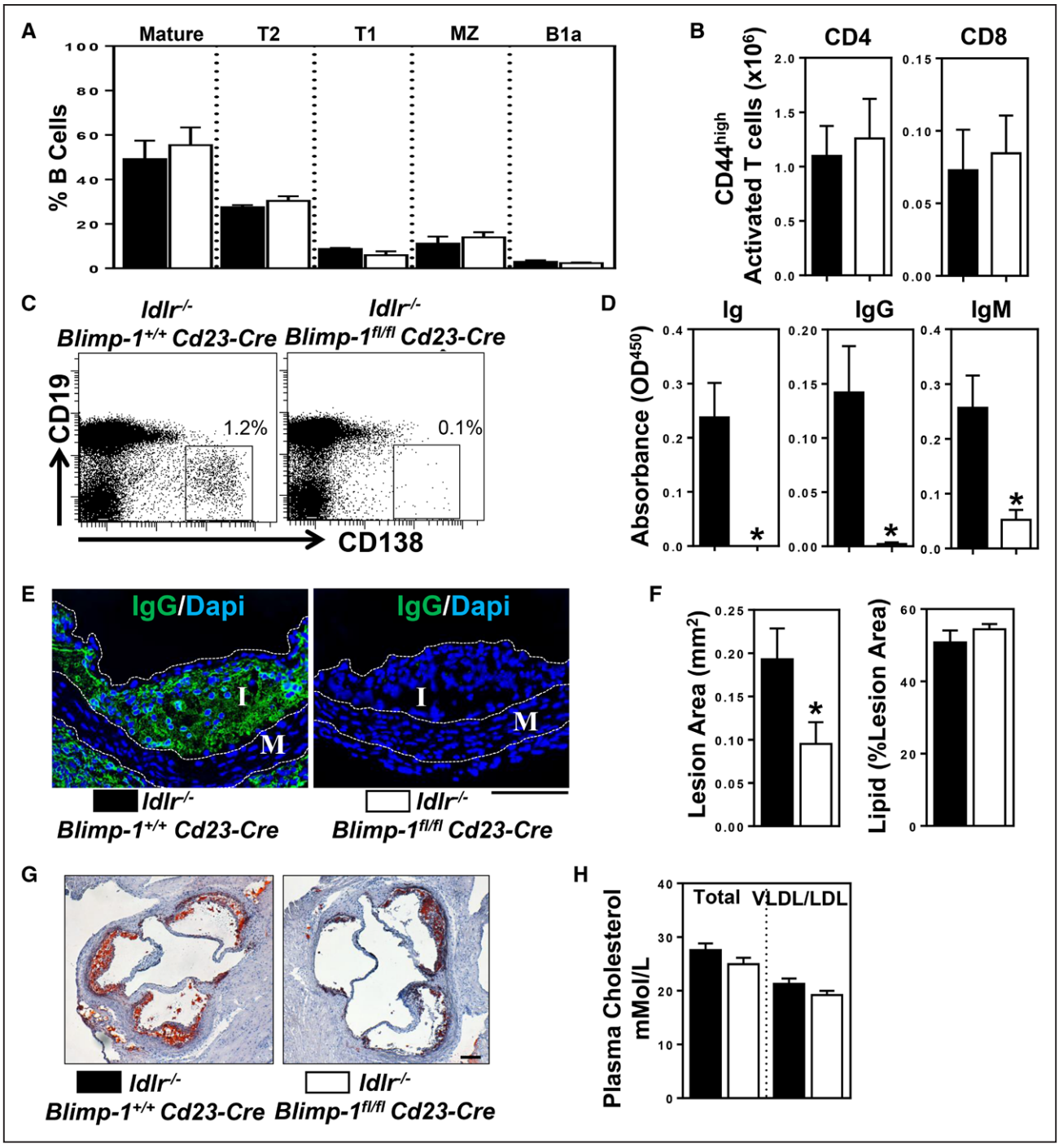

Figure 3. Atherosclerosis reduces in the absence of IgG. Atherogenic mice deficient in plasma cells were generated by transferring *Blimp-1$^{fl/fl}$ Cd23-Cre* bone marrow into irradiated *ldlr$^{-/-}$* mice. Fluorescence-activated cell sorter (FACS) analysis at 4 weeks after bone marrow transplantation, just before commencing the high fat diet (HFD), shows that (**A**) B cell subtypes in spleen are unaffected by Blimp-1 deficiency in B cells. At the end of 8-week HFD feeding, Blimp-1 deletion in B cells of chimeric mice shows (**B**) CD44^{hi}-activated T cells in spleen unaffected, but reduce (**C**) spleen plasma cells by >90% and (**D**) plasma immunoglobulins. Mice without plasma cells also show (**E**) a complete absence of IgG in atherosclerotic lesions and (**F** and **G**) reduced atherosclerosis at aortic sinus, while lesion lipid content ratio is unaffected without affecting total and VLDL/LDL cholesterol in plasma. *P<0.05; results are means±SEM, n=9 to 11 mice per group. *Ldlr$^{-/-}$ Blimp-1$^{fl/fl}$ Cd23-Cre*; mice with B cells selectively deficient in Blimp-1, *Ldlr$^{-/-}$ Blimp-1$^{+/+}$ Cd23-Cre*; control mice. Scale Bar, 100 μm. Blimp indicates B-lymphocyte–induced maturation protein; LDL, low-density lipoprotein; and VLDL, very low–density lipoprotein.

cytokines were detected was administered via tail-vein injection every 2 weeks. After 8 weeks, FACS analysis showed comparable numbers of FO B cells, but no plasma cells in spleens, findings similar to that in mice with Blimp-1-deficient B cells that we described earlier (data not shown). Resultant plasma IgG levels in the 2 groups of mice were similar (P>0.05; Figure 4B), but Athero-IgG deposits accumulated to a much greater extent (≈7-fold) in atherosclerotic lesions than WT-IgG

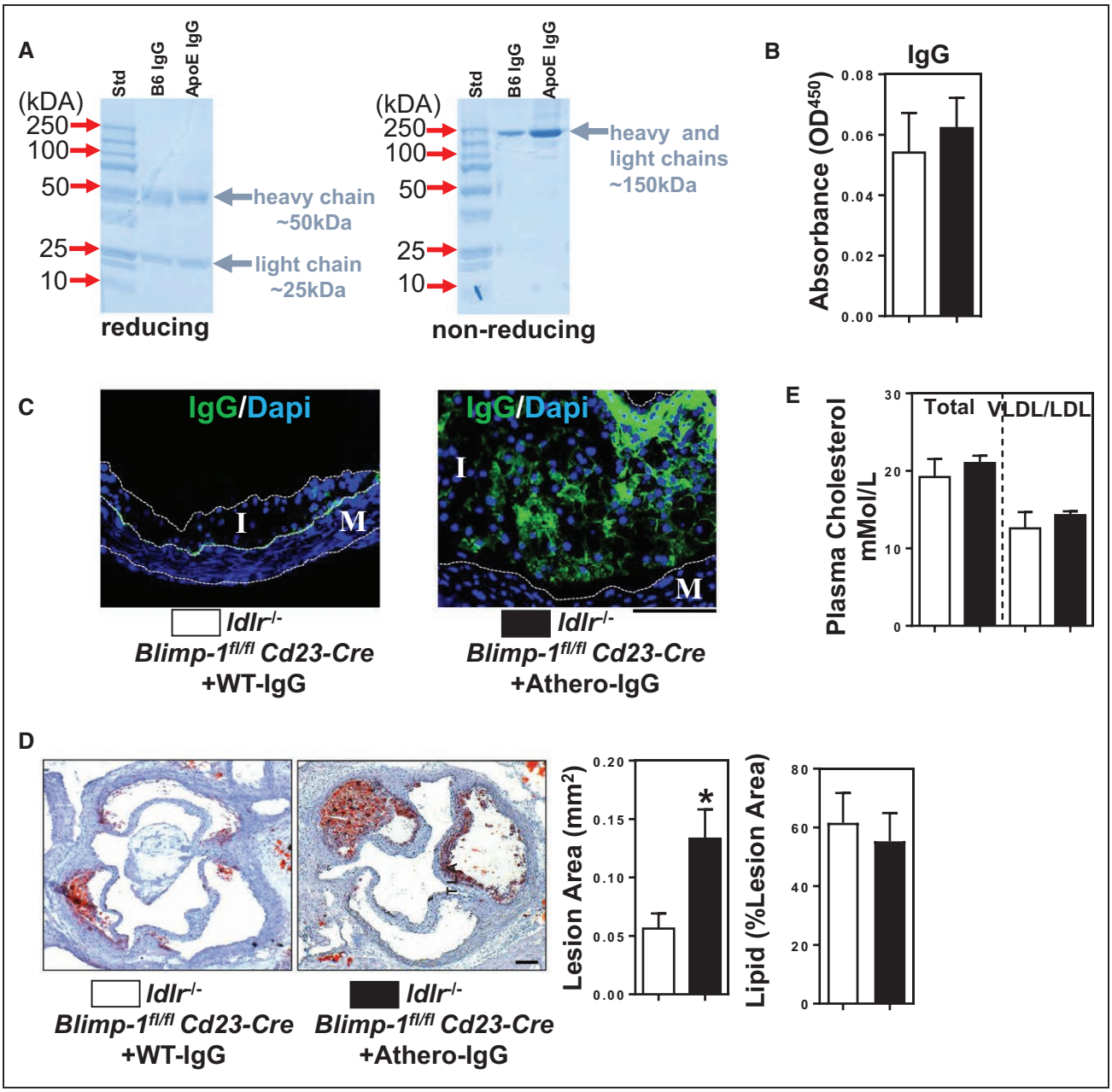

Figure 4. IgG purified from atherosclerotic mice, not from nonatherosclerotic mice, increases atherosclerosis. Plasma IgGs were purified from atherosclerotic mice (high fat diet [HFD]–fed mice) and nonatherosclerotic mice (chow-fed mice). **A**, Gel electrophoresis indicates the purity of IgGs isolated from plasma of mice is greater than 95%. After multiple administrations of IgGs into plasma cell–deficient chimeric mice, (**B**) plasma levels are similar in mice receiving IgGs, but (**C**) IgG deposits are accumulated only in mice that received IgGs from atherosclerotic mice (Athero-IgG) to a far greater extent compared with IgGs from nonatherosclerotic mice (WT-IgG). **D**, Atherosclerotic lesions of mice receiving Athero-IgG are nearly 2.5-fold larger compared with those receiving WT-IgG without affecting (**E**) plasma total cholesterol and VLDL/LDL cholesterols. *P<0.05; results are means±SEM, n=4 to 6 mice per group. *Ldlr*$^{-/-}$ *Blimp-1*$^{fl/fl}$ *Cd23-Cre*; mice with B cells selectively deficient in Blimp-1. Scale Bar, 100 μm. Athero-IgG indicates IgG isolated from plasma of hyperlipidemic/atherosclerotic ApoE$^{-/-}$ mice; Blimp, B-lymphocyte–induced maturation protein; LDL, low-density lipoprotein; VLDL, very low–density lipoprotein; and WT-IgG, IgG isolated from normolipidemic/nonatherosclerotic mice.

deposits (P<0.05; Figure 4C). Furthermore, lesion size in mice receiving Athero-IgG increased 2.4-fold compared with those receiving WT-IgG (P<0.05; Figure 4D); plasma cholesterol levels are unaffected (Figure 4E). It is worthwhile to note that WT-IgG further reduced total lesion area (Figure 4E) compared with those in IgG-deficient mice, suggesting a possible protective role of nonatherosclerotic IgGs in accordance with the literature.[41,42]

IgG From Atherosclerotic Mice Increases Lesion Cell Apoptosis, Necrotic Core Size and Inflammation

IgG immune complexes are known to activate complement and Fcγ receptors inducing apoptosis in target cells.[43,44] As both systems have been implicated in atherosclerosis,[45,46] we examined in gain and loss of function studies whether IgG produced during development of atherosclerosis affects lesion

cell apoptosis and necrosis. After treatment with Athero-IgG lesion, apoptotic cell numbers doubled in *Ldlr*$^{-/-}$ mice whose FO B cells were deficient in Blimp-1 (P<0.05; Figure 5A, right). Athero-IgG also increased lesion necrotic core size, by 80% compared with WT-IgG (P<0.05; Figure 5B, right panel). To confirm these effects of Athero-IgG, we also assessed lesion apoptotic cell numbers and necrotic cores in *Ldlr*$^{-/-}$ mice whose B cells are deficient in Blimp-1, mice whose plasma IgG levels are reduced by 95% (Figure 3F and 3G). In these mice lesion, apoptotic cells were reduced by nearly 53% (P<0.05; Figure 5C, right panel) and necrotic core size by 58%, respectively (P<0.05; Figure 5D, right panel), effects consistent with the Athero-IgG gain of function studies. As necrosis initiates release of HMGB1 (high-mobility group box 1 protein)[47], a potent mediator of sterile inflammation,[48] which promotes atherosclerosis,[49] we examined whether Athero-IgG increases lesion proinflammatory cytokines. Transfer of Athero-IgG into hyperlipidemic *Ldlr*$^{-/-}$ mice with Blimp-1-deficient B cells greatly increased lesion cells expressing TNF-α and IL-1β (P<0.05; Figure 5E and 5F, right panel). Conversely, in chimeric *Ldlr*$^{-/-}$ *Blimp-1*$^{fl/fl}$*Cd23-Cre* mice lesion, TNF-α- and IL-1β-producing cells were reduced (P<0.05; Figure 5G and 5H, right panel). A similar pattern of effects were observed on lesion cells producing RANTES and MCP-1 after transfer of Athero-IgG or preventing the generation of plasma cells (Figure VIII in the online-only Data Supplement).

Discussion

Our study provides the definitive proof that the mechanisms by which FO B cells promote atherosclerosis are critically dependent on their differentiation to plasma cells. This is supported by our findings that the atherogenic effects of FO B cells are also highly dependent on their expression of MHCII and CD40, molecules required for B–T cell interactions and development of germinal center B cells, as well as Blimp-1, a transcriptional factor required for the differentiation of germinal center B cells to plasma cells (Figure 6). FO B cell differentiation to plasma cells seems to be the major mechanism by which FO B cells promote atherosclerosis, given that Blimp-1 deletion in these cells accounts for >80% of the proatherogenic effect of FO B cells on atherosclerosis, when compared with total B cell depletion.[7] Plasma cells seem to mediate their effects on atherosclerosis by producing pathogenic IgGs. Our results are consistent with the report that the Tfh–germinal center B-cell axis is proatherogenic.[50]

Irradiation plus BM transplantation is a widely accepted method in studies on atherosclerosis used to delete specific molecules/cells from *Ldlr*$^{-/-}$ mice[23–25] but results in more rapid development of more inflamed lesions,[51] necessitating the use of appropriate controls.[23–25] As in other studies,[23–25] we also generated appropriate irradiated mixed chimeric controls for our comparative studies of the effects of MHCII, CD40, and Blimp-1 deletions from B cells. Our finding that the ability of FO B cells to promote atherosclerosis is dependent on their expression of MHCII is consistent with our observation that IgG from atherosclerotic mice promotes atherosclerosis. Peptide:MHCII complexes on FO B cells are recognized by antigen-specific CD4$^+$ Tfh cells, stimulating them to make membrane-bound and -secreted factors that stimulate B cell proliferation, form germinal centers, and differentiate into plasma cells. Activated CD4$^+$ T cells but not CD8$^+$ T cells are reduced in spleens of hyperlipidemic mixed chimeric *Ldlr*$^{-/-}$ mice whose B cells do not express MHCII. This reduction in activated CD4$^+$ T cells most likely represents reductions in activated CD4$^+$ Tfh cells rather than CD4$^+$ effector T cells, as B cells in vivo fail to prime naïve T cells[52] but rather stimulate antigen-experienced T cells.[29] The reduction in activated CD4$^+$ T cells is also reflected by the reductions in CD4$^+$IFN-γ$^+$ and CD4$^+$TNF-α$^+$ T cells; both cytokines are important in influencing pathogenic accumulation of Tfh cells and germinal centers.[31,53] Deletion of MHCII on B cells markedly reduces germinal B cell numbers and plasma cells in the spleen, effects consistent with the large reductions in IgGs. Our finding that MHCII deletion on B cells exerts effects on atherosclerosis which are similar in magnitude to those observed with specific B cell deletion[54] indicates that MHCII expressed by FO B cells is a critically important mechanistic pathway by which B2 cells promote atherosclerosis.

The interaction of costimulatory molecules CD40 on FO B cells and CD40L on Tfh cells is also important for high-affinity antibody generation. CD40 expression by FO B cells is necessary for optimal primary B cell responses, including the generation of germinal centers, sustaining antibody production[55] and CD4$^+$ Tfh cell formation.[56] After the large reductions in CD40 expression by B cells in the hyperlipidemic mice, atherosclerosis is also reduced but to a lesser extent than with MHCII deletion, consistent with CD40 on FO B cells being a costimulatory promoter of atherosclerosis; reductions in IgG, spleen germinal B cell, and plasma cell numbers were also less than with MHCII deletion. A recent report has shown that marginal-zone B cells reduce atherosclerosis by mediating Tfh cell development to limiting adaptive immune responses.[57] It is well known that marginal-zone B cells reside both within the marginal zone and follicles.[58] In follicles they present antigens to follicular dendritic cells.[59,60] Collectively, marginal-zone B cells exert both positive and inhibitory effects. Our results tend to suggest that the overall effect of the transient increase in spleen is at best small, based on effects on atherosclerotic lesions 4 weeks after commencing the HFD, where effects on lesions are similar 4 and 8 weeks after commencing the HFD.

Although it is possible that MHCII and CD40 expression by FO B cells contribute to atherosclerosis by mechanisms involving CD4$^+$ T cell activation[61] and increased effector cytokine responses,[62] a common feature of B cell–specific deficiencies in either MHCII or CD40 was the reduction in serum IgG levels. To determine its importance for atherosclerosis, we deleted Blimp-1 in B cells. We used CD23-CRE to specifically delete Blimp-1 from B cells. CD23 is upregulated at the T2 stage of B cell development, and this results in CRE-mediated deletion of Blimp-1 from FO and marginal-zone B cells, as well as most B1 cells.[63,64] This accounts for the large reductions in plasma IgG as well as IgM in mixed chimeric *Ldlr*$^{-/-}$ mice with specific deletion of Blimp-1. Blimp-1 expression by FO B cells is critical for their differentiation into antibody-producing plasma cells. Our findings that deletion of Blimp-1

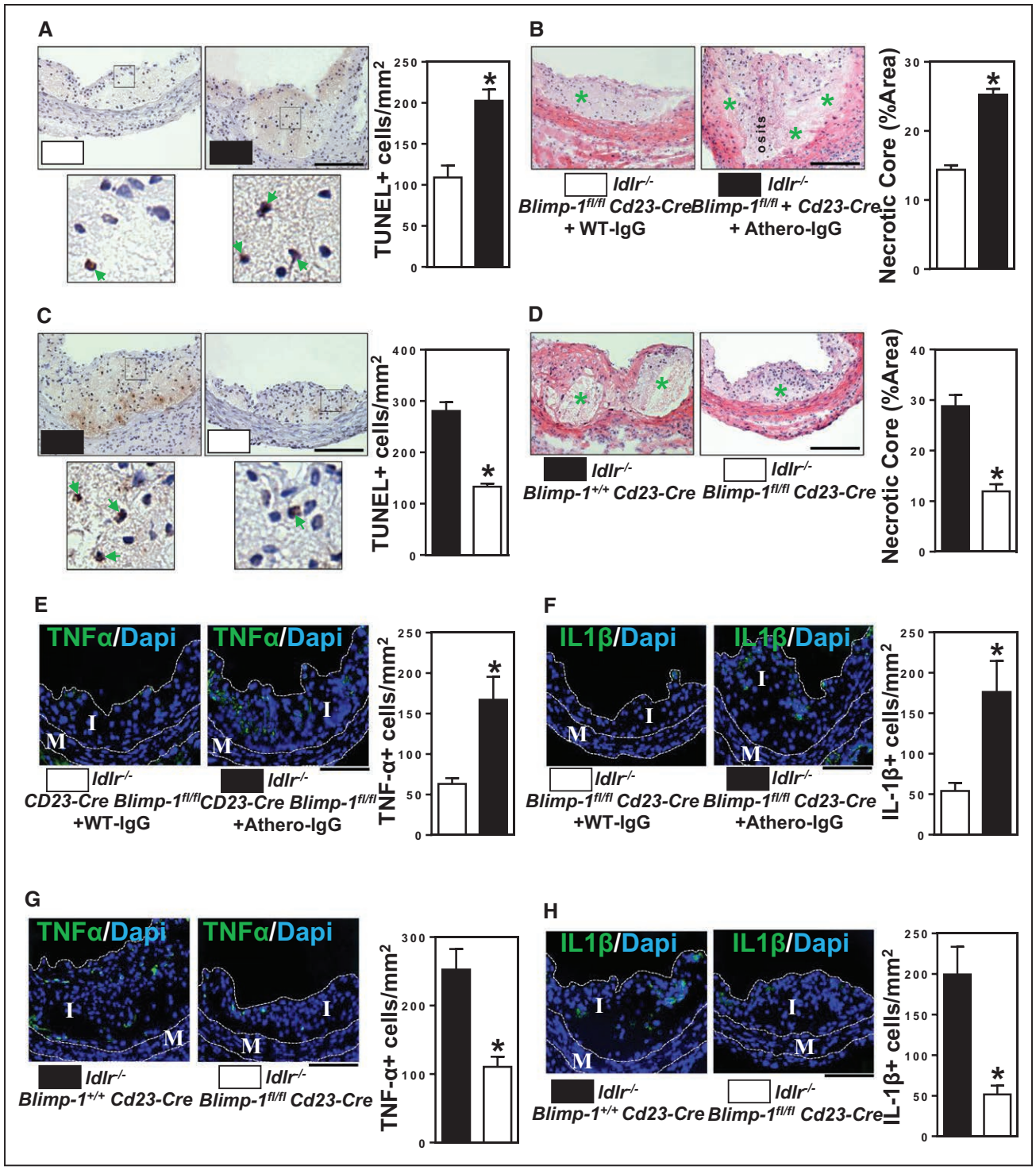

Figure 5. IgG increases cell deaths in atherosclerotic lesions. **A** and **B**, Athero-IgG administration increases lesion apoptotic cell numbers (**A**) and lesion necrotic core size (**B**). **C** and **D**, Deletion of Blimp-1 from B cells, which reduces plasma IgG, attenuates lesion apoptotic cell numbers (**C**) and necrotic core size (**D**). **E** and **F**, Athero-IgG increases numbers of lesion cells expressing TNF-α (tumor necrosis factor-α; **E**) and IL-1β (interleukin-1β; F). **G** and **H**, Deletion of Blimp-1 from B cells, which markedly attenuates IgG production, greatly reduces numbers of lesion cells producing TNF-α (**G**) and IL-1β (**H**). *P<0.05; results are means±SEM, n=4 to 6 mice per group. *Ldlr*$^{-/-}$ *Blimp-1*$^{fl/fl}$ *Cd23-Cre*; mice with B cells selectively deficient in Blimp-1, *Ldlr*$^{-/-}$ *Blimp-1*$^{+/+}$ *Cd23-Cre*; control mice. Scale Bar, 100 μm. Athero-IgG indicates IgG isolated from plasma of hyperlipidemic/atherosclerotic ApoE$^{-/-}$ mice; Blimp, B-lymphocyte–induced maturation protein; LDL, low-density lipoprotein; TUNEL, terminal dUTP nick end-labeling; and WT-IgG, IgG isolated from normolipidemic/nonatherosclerotic mice.

greatly attenuated development of atherosclerosis strongly support the hypothesis that FO B cell differentiation to plasma cells and their production of antibodies, particularly IgG, are critical for the proatherogenic actions of FO B cells. Deletion of Blimp-1 from FO B cells only affects their differentiation to plasma cells, leaving all aspects of B cell MHCII and CD40

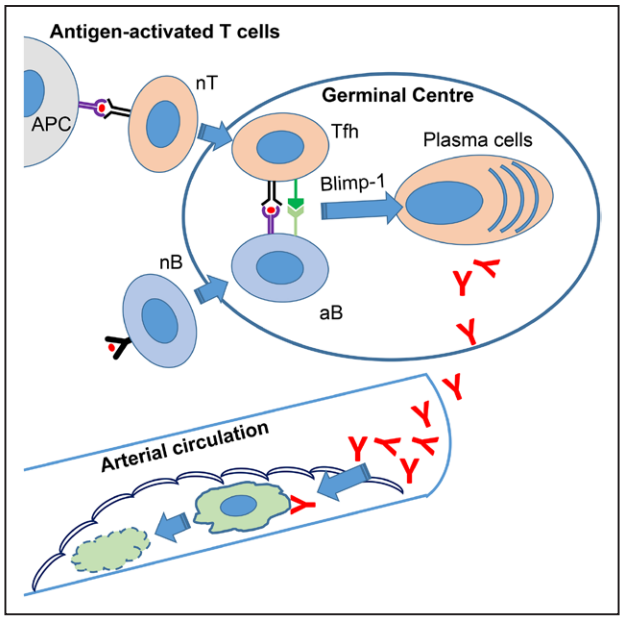

Figure 6. Schematic diagram showing the possible mechanism how B cells can promote atherosclerosis via T cell–dependent manner. Antigen activates naïve T (nT) cells via TCR (T-cell receptor)-dependent manner by professional antigen presenting cells (APC) and naïve B (nB) cells via B-cell receptor. Follicular helper T (Tfh) cells transformed from activated T cells migrate to T–B zone to interact with activated B (aB) cells after which B cells are terminally differentiated into IgG-producing plasma cells. IgG released from plasma cells are deposited in atherosclerotic lesion and induces lesion apoptotic cells via multiple mechanisms. Blimp indicates B-lymphocyte–induced maturation protein.

signaling intact. Blimp-1 deletion in FO B cells of hyperlipidemic chimeric *Ldlr*$^{-/-}$ mice resulted in near complete abolition of Ig and IgG in plasma, suggesting that most IgGs produced by plasma cells before irradiation, to generate chimeric mice, are produced by short-lived radiation-sensitive plasma cells.

Despite associations between circulating immunoglobulins and atherosclerosis being described for >20 years,[16,17] direct proof for a pathogenic role for immunoglobulins is still lacking. Immunoglobulins associated with increased atherosclerosis include IgE,[65] IgA,[66] and IgG,[67] while IgM is generally associated with protection.[4,67] In mixed chimeric mice with targeted depletion of MHCII or CD40, we observed the depletion of these molecules in B1a cells (data not shown), and similar finding has been reported in conditional knockout mouse model to delete MHCII on B cells.[68] The finding that selective deficiency of MHCII and CD40 on B cells does not affect plasma IgM in contrast to the fact that IgM levels plummet down in B cell–selective Blimp-1 deficiency supports that B1a cells, major natural IgM producer in innate-like responses, do not require MHCII and CD40, but Blimp-1 in their secretion of natural antibodies in accordance with literature.[69] It is also important to note that regulatory B cells are heterogeneous in nature, and some of them express neither CD23 nor Blimp-1 for their differentiation/suppressor function.[70,71] Furthermore, activated peritoneal cavity B-1a cells also regulate immune responses by secreting IL-10.[72] We have assessed and found that CD19^{+}CD5^{+} B1a cells (Figure 3A) and B220^{+}CD5^{+}CD23^{-}CD21^{hi} IgM^{+} CD1d cells (data not shown) in spleens of chimeric mice transplanted with CD23-Cre Blimp-1fl/fl BM cells were unaltered compared with their control group, indicating that regulatory B cells are not affected by B cell–specific deletion of Blimp-1. As almost all regulatory B cells modulate immune responses by producing IL-10, we do not expect any effects in our experiments as IL-10-producing B cells, and this is in accordance with literature where B cell–derived IL-10 does not affect atherosclerosis in mice.[73]

Our finding that transfer of IgG purified from atherosclerotic mice into plasma cell and immunoglobulin deficient *Ldlr*$^{-/-}$ *Blimp-1*$^{fl/fl}$*Cd23-Cre* mice accelerated atherosclerosis provides the first definitive proof that IgGs in atherosclerosis are pathogenic. In this study we did not investigate the 2 remaining effector functions—antigen presentation to naïve CD4^{+} T cells or proinflammatory cytokine secretion by B cells because (1) dendritic cells are by far superior antigen-presenting cells compared with B cells,[74] and when CD4^{+} T cell stimulation by antigen-loaded naïve B cells occurs, it results in an unusual T cell phenotype that retains high levels of CD62L and preferentially migrates to T-cell zones in peripheral lymph nodes, contrasting with activation by dendritic cells which downregulate CD62L[75]; (2) activated B cells produce proinflammatory cytokines, and we have already reported that B cells can promote generation of vulnerable plaques by secreting TNF-α (see our article).[10] This cytokine-mediated pathway contributes no >20% to lesion development,[10] and the remainder can be accounted for by IgG-dependent mechanisms described in this article. The transferred IgGs accumulated in atherosclerotic lesions, suggesting that they bind avidly to specific-yet-to-be-defined antigens within developing lesions, contrasting with IgGs from nonatherosclerotic mice which are barely detectable in lesions. The transferred IgGs not only promote progression of atherosclerotic lesions but also affect lesion characteristics, increasing apoptosis/necrotic core size and lesion inflammatory molecules, effects that can be attributed to initial binding of the IgGs to specific target antigens and activation of the complement pathway and cytotoxic immune cells through activation of FcγRs.

The transfer of IgG most likely promoted apoptosis and necrotic core development possibly via NK cell–mediated antibody–dependent cellular cytotoxicity.[76] In this study, we did not specifically study NK cells; they are known to promote necrotic core development in lesions.[77] The process of IgG isolation (extensive wash before eluting IgG from affinity column) prevents a possible contamination of proinflammatory cytokines, and IgGs purified are heterogeneous. Of the major proinflammatory cytokines IL-1α, IL-6, TNF-α, and IL-1β, only IL-1α and IL-6 were shown to bind to IgG purified via ion-exchange chromatography.[78] As both cytokines were not detected in affinity-purified IgG and the plasma half-life of IL-1α and IL-6 in rodents (rats, shorter in mice) is ≈2.5[79] and 3 minutes,[80] respectively, they are extremely unlikely to account for any of the observed IgG effects on atherosclerotic lesions. Antigen–IgG immune complexes accumulated in atherosclerotic lesion can activate NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation in lesion-dominant macrophages through Fcγ receptors for macrophage-derived IL-1β and TNF-α production.[81,82] TNF-α and IL-1β can upregulate both RANTES and MCP-1 through NF-κB activation in an autocrine manner[83,84] and induce apoptosis.[85] Although we have

demonstrated that IgGs from atherosclerotic mice promote atherosclerosis, the specific antigens targeted remain to be identified. Although not specifically studied, our findings that IgG from atherosclerotic mice greatly increase lesion inflammation by increasing expression of proinflammatory cytokines TNF-α and IL-1β and chemokines MCP-1 and RANTES suggest initiation of a complex sterile inflammatory response mediated by release of DAMPs (damage-associated molecular patterns) such as HMGB1 from necrotic cells[47] and activation of the Nlrp3 (nucleotide-binding domain and leucine-rich repeat containing [NLR] protein 3) inflammasome.[52] HMGB1 enhances atherosclerosis development[49] and increases TNF-α and IL-1β expression,[49,86] which may be responsible for the observed elevations in chemokines.[87,88] Among major IgG subclasses, IgG1 is the most abundant subclass induced by soluble and membrane protein antigens, and IgG2 and IgG3 are potent responders to pathogens, with IgG4 being a dominant subclass to allergens.[89] Limitation on current methodology prevents purification of individual IgG subclasses to study their specific roles in atherosclerosis.

In summary, we provide direct evidence that the major mechanism by which FO B cells promote atherosclerosis involves their differentiation to plasma cells. The atherogenic effects of FO B cells are highly dependent on their expression of MHCII, CD40, and Blimp-1. Furthermore, our findings that IgG isolated from atherosclerotic mice greatly promote atherosclerosis confirm their pathogenicity and together with deletion studies indicate that pathogenic IgGs are important contributors to the atherogenic effects of FO B cells. Recently, stable cyclic antibody neutralizing peptides have been developed to specifically target pathogenic antibodies such as those activating cardiac β-1 adrenoceptors.[90] Our study raises the possibility that such approaches may be therapeutically useful to also inhibit the atherogenic effects of B cells without compromising the immune system.

Sources of Funding

This work was supported by grants and fellowships from the National Health and Medical Research Council of Australia (Project Grant 1049416 to A. Kallies), the Sylvia and Charles Viertel Foundation (A. Kallies), the Victorian State Government Operational Infrastructure Support and Australian Government NHMRC Independent Research Institute Infrastructure Support scheme.

Disclosures

None.

References

1. Barquera S, Pedroza-Tobías A, Medina C, Hernández-Barrera L, Bibbins-Domingo K, Lozano R, Moran AE. Global overview of the epidemiology of atherosclerotic cardiovascular disease. *Arch Med Res*. 2015;46:328–338. doi: 10.1016/j.arcmed.2015.06.006.
2. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015;385:117–171. doi: 10.1016/S0140-6736(14)61682-2.
3. Libby P, Lichtman AH, Hansson GK. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity*. 2013;38:1092–1104. doi: 10.1016/j.immuni.2013.06.009.
4. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ. B cells and humoral immunity in atherosclerosis. *Circ Res*. 2014;114:1743–1756. doi: 10.1161/CIRCRESAHA.113.301145.
5. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, Bobik A, Toh BH. B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res*. 2011;109:830–840. doi: 10.1161/CIRCRESAHA.111.248542.
6. Rosenfeld SM, Perry HM, Gonen A, Prohaska TA, Srikakulapu P, Grewal S, Das D, McSkimming C, Taylor AM, Tsimikas S, Bender TP, Witztum JL, McNamara CA. B-1b cells secrete atheroprotective IgM and attenuate atherosclerosis. *Circ Res*. 2015;117:e28–e39. doi: 10.1161/CIRCRESAHA.117.306044.
7. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Toh BH. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. *J Immunol*. 2010;185:4410–4419. doi: 10.4049/jimmunol.1000033.
8. Hosseini H, Li Y, Kanellakis P, Tay C, Cao A, Tipping P, Bobik A, Toh BH, Kyaw T. Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyreactive IgM producing B1a lymphocytes. *Cardiovasc Res*. 2015;106:443–452. doi: 10.1093/cvr/cvv037.
9. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, Swain SL, Lund FE. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol*. 2000;1:475–482. doi: 10.1038/82717.
10. Tay C, Liu YH, Hosseini H, Kanellakis P, Cao A, Peter K, Tipping P, Bobik A, Toh BH, Kyaw T. B-cell-specific depletion of tumour necrosis factor alpha inhibits atherosclerosis development and plaque vulnerability to rupture by reducing cell death and inflammation. *Cardiovasc Res*. 2016;111:385–397. doi: 10.1093/cvr/cvw186.
11. Bosma A, Abdel-Gadir A, Isenberg DA, Jury EC, Mauri C. Lipid-antigen presentation by CD1d(+) B cells is essential for the maintenance of invariant natural killer T cells. *Immunity*. 2012;36:477–490. doi: 10.1016/j.immuni.2012.02.008.
12. Archambault AS, Carrero JA, Barnett LG, McGee NG, Sim J, Wright JO, Raabe T, Chen P, Ding H, Allenspach EJ, Dragatsis I, Laufer TM, Wu GF. Cutting edge: conditional MHC class II expression reveals a limited role for B cell antigen presentation in primary and secondary CD4 T cell responses. *J Immunol*. 2013;191:545–550. doi: 10.4049/jimmunol.1201598.
13. Noorchashm H, Reed AJ, Rostami SY, Mozaffari R, Zekavat G, Koeberlein B, Caton AJ, Naji A. B cell-mediated antigen presentation is required for the pathogenesis of acute cardiac allograft rejection. *J Immunol*. 2006;177:7715–7722.
14. King C. New insights into the differentiation and function of T follicular helper cells. *Nat Rev Immunol*. 2009;9:757–766. doi: 10.1038/nri2644.
15. Hoffman W, Lakkis FG, Chalasani G. B cells, antibodies, and more. *Clin J Am Soc Nephrol*. 2016;11:137–154. doi: 10.2215/CJN.09430915.
16. Xu Q, Willeit J, Marosi M, Kleindienst R, Oberhollenzer F, Kiechl S, Stulnig T, Luef G, Wick G. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. *Lancet*. 1993;341:255–259.
17. Xu Q, Kiechl S, Mayr M, Metzler B, Egger G, Oberhollenzer F, Willeit J, Wick G. Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis: clinical significance determined in a follow-up study. *Circulation*. 1999;100:1169–1174.
18. Lopes-Virella MF, Virella G. Pathogenic role of modified LDL antibodies and immune complexes in atherosclerosis. *J Atheroscler Thromb*. 2013;20:743–754.
19. Tellier J, Shi W, Minnich M, Liao Y, Crawford S, Smyth GK, Kallies A, Busslinger M, Nutt SL. Blimp-1 controls plasma cell function through the regulation of immunoglobulin secretion and the unfolded protein response. *Nat Immunol*. 2016;17:323–330. doi: 10.1038/ni.3348.
20. Cosgrove D, Gray D, Dierich A, Kaufman J, Lemeur M, Benoist C, Mathis D. Mice lacking MHC class II molecules. *Cell*. 1991;66:1051–1066.
21. Malin S, McManus S, Cobaleda C, Novatchkova M, Delogu A, Bouillet P, Strasser A, Busslinger M. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. *Nat Immunol*. 2010;11:171–179. doi: 10.1038/ni.1827.
22. Kallies A, Xin A, Belz GT, Nutt SL. Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity*. 2009;31:283–295. doi: 10.1016/j.immuni.2009.06.021.
23. Boisvert WA, Spangenberg J, Curtiss LK. Role of leukocyte-specific LDL receptors on plasma lipoprotein cholesterol and atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*. 1997;17:340–347.
24. Murphy AJ, Bijl N, Yvan-Charvet L, Welch CB, Bhagwat N, Reheman A, Wang Y, Shaw JA, Levine RL, Ni H, Tall AR, Wang N. Cholesterol efflux in megakaryocyte progenitors suppresses platelet production and thrombocytosis. *Nat Med*. 2013;19:586–594. doi: 10.1038/nm.3150.

25. Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RV Jr. Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest*. 2001;107:163–171. doi: 10.1172/JCI10310.
26. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol*. 2002;3:944–950. doi: 10.1038/ni833.
27. Daugherty A, Tall AR, Daemen MJAP, Falk E, Fisher EA, García-Cardeña G, Lusis AJ, Owens AP III, Rosenfeld ME, Virmani R; American Heart Association Council on Arteriosclerosis, Thrombosis and Vascular Biology; and Council on Basic Cardiovascular Sciences. Recommendation on design, execution, and reporting of animal atherosclerosis studies: a scientific statement from the American Heart Association. *Arterioscler Thromb Vasc Biol*. 2017;37:e131–e157. doi: 10.1161/ATV.0000000000000062.
28. Li Y, To K, Kanellakis P, Hosseini H, Deswaerte V, Tipping P, Smyth MJ, Toh BH, Bobik A, Kyaw T. CD4+ natural killer T cells potently augment aortic root atherosclerosis by perforin- and granzyme B-dependent cytotoxicity. *Circ Res*. 2015;116:245–254. doi: 10.1161/CIRCRESAHA.116.304734.
29. Barnett LG, Simkins HM, Barnett BE, Korn LL, Johnson AL, Wherry EJ, Wu GF, Laufer TM. B cell antigen presentation in the initiation of follicular helper T cell and germinal center differentiation. *J Immunol*. 2014;192:3607–3617. doi: 10.4049/jimmunol.1301284.
30. Maehr R, Kraus M, Ploegh HL. Mice deficient in invariant-chain and MHC class II exhibit a normal mature B2 cell compartment. *Eur J Immunol*. 2004;34:2230–2236. doi: 10.1002/eji.200425246.
31. Lee SK, Silva DG, Martin JL, Pratama A, Hu X, Chang PP, Walters G, Vinuesa CG. Interferon-γ excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. *Immunity*. 2012;37:880–892. doi: 10.1016/j.immuni.2012.10.010.
32. Fujii T, Okada M, Mimori T, Craft J. The transmembrane form of TNF-alpha drives autoantibody production in the absence of CD154: studies using MRL/Mp-Fas(lpr) mice. *Clin Exp Immunol*. 2002;130:224–232.
33. Han S, Dillon SR, Zheng B, Shimoda M, Schlissel MS, Kelsoe G. V(D)J recombinase activity in a subset of germinal center B lymphocytes. *Science*. 1997;278:301–305.
34. Laszlo G, Hathcock KS, Dickler HB, Hodes RJ. Characterization of a novel cell-surface molecule expressed on subpopulations of activated T and B cells. *J Immunol*. 1993;150:5252–5262.
35. Kawabe T, Naka T, Yoshida K, Tanaka T, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T, Kikutani H. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity*. 1994;1:167–178.
36. Haase C, Michelsen BK, Jørgensen TN. CD40 is necessary for activation of naïve T cells by a dendritic cell line *in vivo* but not *in vitro*. *Scand J Immunol*. 2004;59:237–245. doi: 10.1111/j.0300-9475.2004.01390.x.
37. Fuleihan R, Ramesh N, Geha RS. Role of CD40-CD40-ligand interaction in Ig-isotype switching. *Curr Opin Immunol*. 1993;5:963–967.
38. Shimotakahara S, Mayberg MR. Gamma irradiation inhibits neointimal hyperplasia in rats after arterial injury. *Stroke*. 1994;25:424–428.
39. Boettger T, Beetz N, Kostin S, Schneider J, Krüger M, Hein L, Braun T. Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. *J Clin Invest*. 2009;119:2634–2647. doi: 10.1172/JCI38864.
40. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity*. 2003;19:607–620.
41. Nicoletti A, Kaveri S, Caligiuri G, Bariéty J, Hansson GK. Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. *J Clin Invest*. 1998;102:910–918. doi: 10.1172/JCI119892.
42. Persson L, Borén J, Nicoletti A, Hansson GK, Pekna M. Immunoglobulin treatment reduces atherosclerosis in apolipoprotein E-/- low-density lipoprotein receptor-/- mice via the complement system. *Clin Exp Immunol*. 2005;142:441–445. doi: 10.1111/j.1365-2249.2005.02954.x.
43. Nauta AJ, Daha MR, Tijsma O, van de Water B, Tedesco F, Roos A. The membrane attack complex of complement induces caspase activation and apoptosis. *Eur J Immunol*. 2002;32:783–792.
44. Wilson NS, Yang B, Yang A, et al. An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell*. 2011;19:101–113. doi: 10.1016/j.ccr.2010.11.012.
45. Lewis RD, Jackson CL, Morgan BP, Hughes TR. The membrane attack complex of complement drives the progression of atherosclerosis in apolipoprotein E knockout mice. *Mol Immunol*. 2010;47:1098–1105. doi: 10.1016/j.molimm.2009.10.035.
46. Hernández-Vargas P, Ortiz-Muñoz G, López-Franco O, Suzuki Y, Gallego-Delgado J, Sanjuán G, Lázaro A, López-Parra V, Ortega L, Egido J, Gómez-Guerrero C. Fcgamma receptor deficiency confers protection against atherosclerosis in apolipoprotein E knockout mice. *Circ Res*. 2006;99:1188–1196. doi: 10.1161/01.RES.0000250556.07796.6c.
47. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*. 2002;418:191–195. doi: 10.1038/nature00858.
48. Tsung A, Tohme S, Billiar TR. High-mobility group box-1 in sterile inflammation. *J Intern Med*. 2014;276:425–443. doi: 10.1111/joim.12276.
49. Kanellakis P, Agrotis A, Kyaw TS, Koulis C, Ahrens I, Mori S, Takahashi HK, Liu K, Peter K, Nishibori M, Bobik A. High-mobility group box protein 1 neutralization reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice. *Arterioscler Thromb Vasc Biol*. 2011;31:313–319. doi: 10.1161/ATVBAHA.110.218669.
50. Clement M, Guedj K, Andreata F, et al. Control of the T follicular helper-germinal center B-cell axis by $CD8^+$ regulatory T cells limits atherosclerosis and tertiary lymphoid organ development. *Circulation*. 2015;131:560–570. doi: 10.1161/CIRCULATIONAHA.114.010988.
51. Schiller NK, Kubo N, Boisvert WA, Curtiss LK. Effect of gamma-irradiation and bone marrow transplantation on atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2001;21:1674–1680.
52. Iyer SS, Pulskens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, Eisenbarth SC, Florquin S, Flavell RA, Leemans JC, Sutterwala FS. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci USA*. 2009;106:20388–20393. doi: 10.1073/pnas.0908698106.
53. Taniguchi T, Takata M, Ikeda A, Momotani E, Sekikawa K. Failure of germinal center formation and impairment of response to endotoxin in tumor necrosis factor alpha-deficient mice. *Lab Invest*. 1997;77:647–658.
54. Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, MacKay F, Tipping P, Bobik A, Toh BH. Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PLoS One*. 2012;7:e29371. doi: 10.1371/journal.pone.0029371.
55. Lee BO, Moyron-Quiroz J, Rangel-Moreno J, Kusser KL, Hartson L, Sprague F, Lund FE, Randall TD. CD40, but not CD154, expression on B cells is necessary for optimal primary B cell responses. *J Immunol*. 2003;171:5707–5717.
56. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, Tangye SG. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity*. 2010;33:241–253. doi: 10.1016/j.immuni.2010.07.015.
57. Nus M, Sage AP, Lu Y, et al. Marginal zone B cells control the response of follicular helper T cells to a high-cholesterol diet. *Nat Med*. 2017;23:601–610. doi: 10.1038/nm.4315.
58. Zhou Z, Niu H, Zheng YY, Morel L. Autoreactive marginal zone B cells enter the follicles and interact with CD4+ T cells in lupus-prone mice. *BMC Immunol*. 2011;12:7. doi: 10.1186/1471-2172-12-7.
59. Ferguson AR, Youd ME, Corley RB. Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. *Int Immunol*. 2004;16:1411–1422. doi: 10.1093/intimm/dxh142.
60. Cinamon G, Zachariah MA, Lam OM, Foss FW Jr, Cyster JG. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat Immunol*. 2008;9:54–62. doi: 10.1038/ni1542.
61. Ronchese F, Hausmann B. B lymphocytes *in vivo* fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. *J Exp Med*. 1993;177:679–690.
62. Duddy ME, Alter A, Bar-Or A. Distinct profiles of human B cell effector cytokines: a role in immune regulation? *J Immunol*. 2004;172:3422–3427.
63. Chung JB, Sater RA, Fields ML, Erikson J, Monroe JG. CD23 defines two distinct subsets of immature B cells which differ in their responses to T cell help signals. *Int Immunol*. 2002;14:157–166.
64. Montecino-Rodriguez E, Dorshkind K. Formation of B-1 B cells from neonatal B-1 transitional cells exhibits NF-κB redundancy. *J Immunol*. 2011;187:5712–5719. doi: 10.4049/jimmunol.1102416.
65. Wang J, Cheng X, Xiang MX, et al. IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in $Apoe^{-/-}$ mice. *J Clin Invest*. 2011;121:3564–3577. doi: 10.1172/JCI46028.
66. Muscari A, Bozzoli C, Gerratana C, Zaca' F, Rovinetti C, Zauli D, La Placa M, Puddu P. Association of serum IgA and C4 with severe atherosclerosis. *Atherosclerosis*. 1988;74:179–186.
67. Tsimikas S, Brilakis ES, Lennon RJ, Miller ER, Witztum JL, McConnell JP, Kornman KS, Berger PB. Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. *J Lipid Res*. 2007;48:425–433. doi: 10.1194/jlr.M600361-JLR200.

68. Shimoda M, Li T, Pihkala JP, Koni PA. Role of MHC class II on memory B cells in post-germinal center B cell homeostasis and memory response. *J Immunol*. 2006;176:2122–2133.
69. Savitsky D, Calame K. B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. *J Exp Med*. 2006;203:2305–2314. doi: 10.1084/jem.20060411.
70. Mauri C, Menon M. The expanding family of regulatory B cells. *Int Immunol*. 2015;27:479–486. doi: 10.1093/intimm/dxv038.
71. Zhang X. Regulatory functions of innate-like B cells. *Cell Mol Immunol*. 2013;10:113–121. doi: 10.1038/cmi.2012.63.
72. Margry B, Kersemakers SC, Hoek A, Arkesteijn GJ, Wieland WH, van Eden W, Broere F. Activated peritoneal cavity B-1a cells possess regulatory B cell properties. *PLoS One*. 2014;9:e88869. doi: 10.1371/journal.pone.0088869.
73. Sage AP, Nus M, Baker LL, Finigan AJ, Masters LM, Mallat Z. Regulatory B cell-specific interleukin-10 is dispensable for atherosclerosis development in mice. *Arterioscler Thromb Vasc Biol*. 2015;35:1770–1773. doi: 10.1161/ATVBAHA.115.305568.
74. Lassila O, Vainio O, Matzinger P. Can B cells turn on virgin T cells? *Nature*. 1988;334:253–255. doi: 10.1038/334253a0.
75. Reichardt P, Dornbach B, Rong S, Beissert S, Gueler F, Loser K, Gunzer M. Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. *Blood*. 2007;110:1519–1529. doi: 10.1182/blood-2006-10-053793.
76. Wang W, Erbe AK, Hank JA, Morris ZS, Sondel PM. NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. *Front Immunol*. 2015;6:368. doi: 10.3389/fimmu.2015.00368.
77. Selathurai A, Deswaerte V, Kanellakis P, Tipping P, Toh BH, Bobik A, Kyaw T. Natural killer (NK) cells augment atherosclerosis by cytotoxic-dependent mechanisms. *Cardiovasc Res*. 2014;102:128–137. doi: 10.1093/cvr/cvu016.
78. Svenson M, Hansen MB, Bendtzen K. Binding of cytokines to pharmaceutically prepared human immunoglobulin. *J Clin Invest*. 1993;92:2533–2539. doi: 10.1172/JCI116862.
79. Poole S, Bird TA, Selkirk S, Gaines-Das RE, Choudry Y, Stephenson SL, Kenny AJ, Saklatvaa J. Fate of injected interleukin 1 in rats: sequestration and degradation in the kidney. *Cytokine*. 1990;2:416–422.
80. Castell JV, Geiger T, Gross V, Andus T, Walter E, Hirano T, Kishimoto T, Heinrich PC. Plasma clearance, organ distribution and target cells of interleukin-6/hepatocyte-stimulating factor in the rat. *Eur J Biochem*. 1988;177:357–361.
81. Lentsch AB, Czermak BJ, Bless NM, Ward PA. NF-kappaB activation during IgG immune complex-induced lung injury: requirements for TNF-alpha and IL-1beta but not complement. *Am J Pathol*. 1998;152:1327–1336.
82. Cao S, Theodore S, Standaert DG. Fcγ receptors are required for NF-κB signaling, microglial activation and dopaminergic neurodegeneration in an AAV-synuclein mouse model of Parkinson's disease. *Mol Neurodegener*. 2010;5:42. doi: 10.1186/1750-1326-5-42.
83. Ciesielski CJ, Andreakos E, Foxwell BM, Feldmann M. TNFalpha-induced macrophage chemokine secretion is more dependent on NF-kappaB expression than lipopolysaccharides-induced macrophage chemokine secretion. *Eur J Immunol*. 2002;32:2037–2045.
84. Andoh A, Takaya H, Saotome T, Shimada M, Hata K, Araki Y, Nakamura F, Shintani Y, Fujiyama Y, Bamba T. Cytokine regulation of chemokine (IL-8, MCP-1, and RANTES) gene expression in human pancreatic periacinar myofibroblasts. *Gastroenterology*. 2000;119:211–219.
85. Ye L, Huang Y, Zhao L, Li Y, Sun L, Zhou Y, Qian G, Zheng JC. IL-1β and TNF-α induce neurotoxicity through glutamate production: a potential role for neuronal glutaminase. *J Neurochem*. 2013;125:897–908. doi: 10.1111/jnc.12263.
86. Chen XL, Sun L, Guo F, Wang F, Liu S, Liang X, Wang RS, Wang YJ, Sun YX. High-mobility group box-1 induces proinflammatory cytokines production of Kupffer cells through TLRs-dependent signaling pathway after burn injury. *PLoS One*. 2012;7:e50668. doi: 10.1371/journal.pone.0050668.
87. Ozaki K, Hanazawa S, Takeshita A, Chen Y, Watanabe A, Nishida K, Miyata Y, Kitano S. Interleukin-1 beta and tumor necrosis factor-alpha stimulate synergistically the expression of monocyte chemoattractant protein-1 in fibroblastic cells derived from human periodontal ligament. *Oral Microbiol Immunol*. 1996;11:109–114.
88. Ammit AJ, Lazaar AL, Irani C, O'Neill GM, Gordon ND, Amrani Y, Penn RB, Panettieri RA Jr. Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and beta-agonists. *Am J Respir Cell Mol Biol*. 2002;26:465–474. doi: 10.1165/ajrcmb.26.4.4681.
89. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol*. 2014;5:520. doi: 10.3389/fimmu.2014.00520.
90. Münch G, Boivin-Jahns V, Holthoff HP, Adler K, Lappo M, Truöl S, Degen H, Steiger N, Lohse MJ, Jahns R, Ungerer M. Administration of the cyclic peptide COR-1 in humans (phase I study): ex vivo measurements of anti-β1-adrenergic receptor antibody neutralization and of immune parameters. *Eur J Heart Fail*. 2012;14:1230–1239. doi: 10.1093/eurjhf/hfs118.

Highlights

- Follicular B cells interact with T-follicular helper cells via MHCII (major histocompatibility II)- and CD40 (cluster of differentiation 40)-dependent manners to become IgG-producing plasma cells.
- B- and T-cell interaction is critically important for follicular B cell's atherogenicity.
- Atherosclerosis is ameliorated in the absence of atherogenic IgGs and their restoration augments atherosclerosis.

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Follicular B Cells Promote Atherosclerosis via T Cell–Mediated Differentiation Into Plasma Cells and Secreting Pathogenic Immunoglobulin G

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Arterioscler Thromb Vasc Biol. 2018;38:e71-e84; originally published online March 29, 2018; doi: 10.1161/ATVBAHA.117.310678

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/38/5/e71

Data Supplement (unedited) at:
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SUPPLEMENTARY MATERIAL

B cells Promote Atherosclerosis via T Cell Mediated Differentiation into Plasma Cells and Secreting Pathogenic Immunoglobulin G

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Supplementary tables

Supplementary Table I. Mixed chimeric mouse generation strategy.

	Control (1)		**Test (2)**		**Alternative control (3)**	
	Bone Marrow cells (ratio)					
	80% WT + 20% (Gene$^{-/-}$)		80% µMT + 20% (Gene$^{-/-}$)		80% µMT + 20% (WT)	
	Immune Cell Reconstitution					
	WT	Gene$^{-/-}$	WT	Gene$^{-/-}$	WT	Gene$^{-/-}$
B cells	80%	20%	-	100%	100%	-
APCs	80%	20%	80%	20%	100%	-
T cells	80%	20%	80%	20%	100%	-
Other non-B cells	80%	20%	80%	20%	100%	-

Three possible mixed chimeric mouse models are available and widely used to study the role of cell-specific gene expression. In atherosclerosis, many immune cells such as macrophages, monocytes, dendritic cells are involved and contribute to its pathogenesis. Therefor control mouse model (1) and test mouse model (2) are used to generate a comparable non-B cell composition. Alternative control mouse model (3) has also been generated and included in this study. (see text for detail)

Supplementary Table II. Atherosclerotic plaque characteristics.

	Control mice	Test mice	p value
B cell-expressing MHCII			
Macrophage area ratio	65.8 ± 3.7 %	67.6 ± 4.1 %	n.s
CD19+ B cells	53 ± 4 cells/mm^2	62 ± 2 cells/mm^2	n.s
CD3+ T cells	88 ± 7 cells/mm^2	88 ± 3 cells/mm^2	n.s
Collagen area ratio	8.8 ± 0.7 %	8.0 ± 0.9 %	n.s
IgG deposit area ratio	23.3 ± 2.2 %	7.0 ± 1.12 %	*
B cell-expressing CD40			
Macrophage area ratio	69.2 ± 4.0 %	62.1 ± 5.1 %	n.s
CD19+ B cells	53 ± 4 cells/mm^2	60 ± 8 cells/mm^2	n.s
CD3+ T cells	83 ± 5 cells/mm^2	94 ± 3 cells/mm^2	n.s
Collagen area ratio	11.0 ± 1.2 %	11.0 ± 1.2 %	n.s
IgG deposit area ratio	26.5 ± 2.1 %	6.2 ± 0.4 %	*
B cell-expressing Blimp-1			
Macrophage area ratio	70.1 ± 4.6 %	68.1 ± 9.1 %	n.s
CD19+ B cells	42 ± 1 cells/mm^2	53 ± 8 cells/mm^2	n.s
CD3+ T cells	100 ± 6 cells/mm^2	96 ± 7 cells/mm^2	n.s
Collagen area ratio	7.9 ± 1.1 %	6.6 ± 1.5 %	n.s
IgG deposit area ratio	26.9 ± 2.6 %	0.4 ± 0.1 %	*
B cell-expressing Blimp-1 + IgG			
Macrophage area ratio	65.4 ± 4.3 %	63.8 ± 6.2 %	n.s
CD19+ B cells	60 ± 1 cells/mm^2	64± 5 cells/mm^2	n.s
CD3+ T cells	104 ± 5 cells/mm^2	105 ± 8 cells/mm^2	n.s
IgG deposit area ratio	2.3 ± 0.4 %	27.9 ± 1.9 %	*

Cellular contents (macrophages, CD3+ T cells and CD19+ B cells), collagen contents and IgG deposits were assessed in all experiments using immunohistochemical, immunofluorescence and histological staining. Data on macrophages, collagen and IgG deposits were presented as % of lesion area. Results are means ± SEM, n = 9-10 mice per group. n.s; not significant, *P < 0.05.

Supplementary Figure Legends

Supplementary Figure I. B cell-selective gene deficiency of MHC II or CD40 does not affect macrophages. Chimeric mice with B cell-specific deficiency of MHCII or CD40 were generated using mixed chimeric strategy. At the completion of experiment, FACS analysis showed that (A-B) MHC II and (C-D) CD40 expression on macrophages was not affected in mice with B cell-specific deletion of MHCII and CD40 molecules respectively. results are means ± SEM, n = 9-10 mice per group. $MHCII0^{+/+}$ B; mice with B cells expressing MHCII, $MHCII^{-/-}$ B; mice with B cells selectively deficient in MHCII, $CD40^{+/+}$ B; mice with B cells expressing CD40, $CD40^{-/-}$ B; mice with B cells selectively deficient in CD40.

Supplementary Figure II. B cell-selective deficiency of MHC II does not affect B cell subsets. Spleen B cells were stained with different fluorochrome labelled antibodies and FACS data were acquired using BD Facs Canto II. (A) During analysis, lymphocyte-gated cells were grouped into CD23+ and CD23- cells both of which were further analysed for CD21 and IgM positivity. Mature (follicular) B cells; CD23+CD21+ IgM^{lo}, T1; CD23- CD21- IgM+, T2; CD23+ CD21+ IgM^{hi}, MZV; CD23- CD21+ IgM+. B1a cells were defined as CD19+ CD5+ cells. FACS analysis showed that B cell-selective MHCII deficiency did not affect (B) spleen B cell subsets and (B) spleen lymphocytes or monocytes/macrophages, measured 4 week after bone marrow transplantation, just before commencement of the HFD. Black; control mice, White; mice with B cells selectively deficient in MHCII, results are means ± SEM, n = 8-9 mice per group.

Supplementary Figure III. B cell-selective deficiency of MHCII molecule reduces spleen CD4 T cells. FACS analysis indicates that MHC II deficiency (A) Activated spleen CD4+ but not CD8+ T cells are reduced in mixed chimeric *Ldlr*$^{-/-}$ mice with B cells deficient in MHC II and (B) Spleen CD4+ T cells expressing IFN-ɣ or TNF-α are reduced in *Ldlr*$^{-/-}$ mice with B cells deficient in MHC II. (C) Plasma VLDL/LDL cholesterol is unaffected by MHC II deletion in B cells. *$P < 0.05$; results are means ± SEM, n = 13-15 per group. $MHCII^{-/-}$ B; mice with B cells selectively deficient in MHCII, $MHCII^{+/+}$ B; control mice.

Supplementary Figure IV. B cell-selective deficiency of MHCII or CD40 reduces T follicular helper T cells. *Ldlr*$^{-/-}$ mixed chimeric mice with B cells selectively deficient in either MHCII or CD40 were killed after 8 week HFD feeding and single cell suspension prepared from spleens were stained for CD4 T follicular helper (Tfh) cells as determined by CD4, CD44, PD-1 and Bcl6 antibodies. (A) Schematic diagram showing Tfh cell assessment. Parentheses indicate percentage of gated cells. FACS analysis showed that (B) MHCII deficiency and (C) CD40 deficiency limited on B cells reduced CD4 Tfh cells. results are means ± SEM, Black; control, White; test, n = 8-9 mice per group.

Supplementary Figure V. Atherosclerosis is reduced in B cells deficient in CD40 assessed at 0, 4 weeks and 8 weeks high fat diet. (A) Assessment at aortic sinus at weeks 0, 4 and 8 weeks of HFD and at innominate artery at 8 weeks of HFD indicates a reduction in atherosclerosis in mice with B cells selectively deficient in CD40 without affecting (B) spleen lymphocyte or monocyte/macrophages populations and (C) plasma IgM levels. But (D) MZ B cells are increased 4 weeks after bone marrow transplantation.

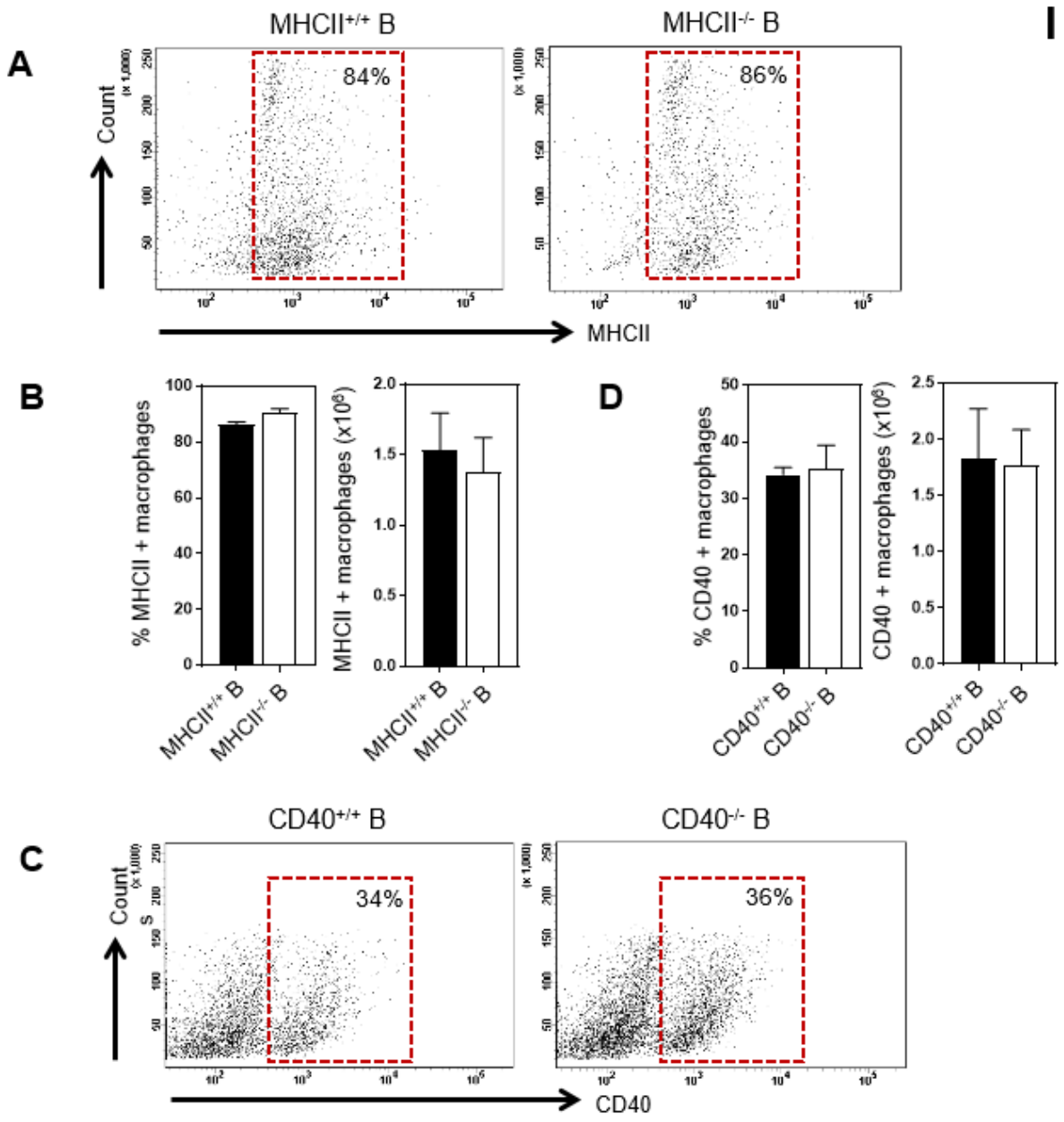
However (E) all B cell subsets including MZ B cells are similar between test and control mice at the end of experiment. Black; control mice, White; mice with B cells selectively deficient in CD40, results are means ± SEM, n = 8-9 mice per group. Scale Bar, 100μm.

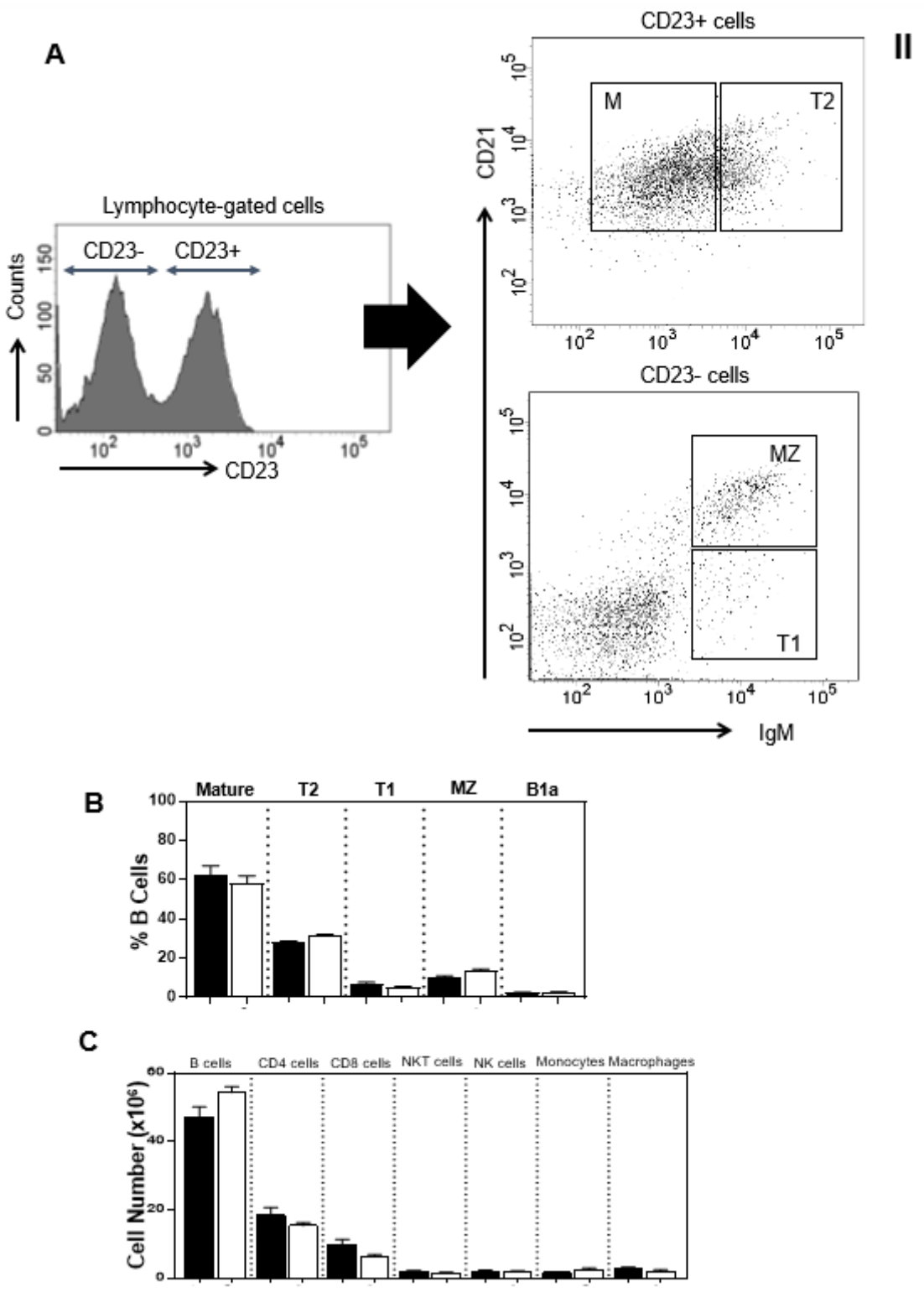
Supplementary Figure VI. B cell-selective deficiency of CD40 molecule reduces spleen IFN-ɣ-producing CD4 T cells. (A) Spleen CD4+IFN-ɣ+ cells are reduced in mixed chimeric *Ldlr*$^{-/-}$ mice with reduced CD40 expression, however (B) total plasma cholesterol and VLDL/LDL-cholesterol are unaffected by modulating CD40 expression in B cells. *P < 0.05; results are means ± SEM, n = 15-17 mice per group. CD40$^{-/-}$ B; mice with B cells selectively deficient in CD40, CD40$^{+/+}$ B; control mice.

Supplementary Figure VII. Total Body Irradiation does not affect smooth muscle content in tunica media. Aortic sinuses were collected at different time points from mixed chimeric *Ldlr*$^{-/-}$ mice with B cells selectively deficient in CD40 molecule and also collected from C57Bl/6 and HFD-fed ApoE$^{-/-}$ mice and C57Bl/6 mice that were not exposed to total Body Irradiation. Smooth muscle cells in tunica media at aortic sinus were determined by staining immunohistochemically with anti-α-actin antibody and expressed them after correction to medial areas. ND, not done, results are means ± SEM, n = 6-8 mice per group.

Supplementary Figure VIII. IgG increases Rantes+ and MCP-1 cells in lesions (A & B) Transfer of Athero-IgG but not WT-IgG increases lesions cells producing RANTES and MCP-1. (C & D) In lesions of Chimeric *Ldlr*$^{-/-}$ *Blimp-1*$^{fl/fl}$*Cd23-Cre* mice cell producing RANTES and MCP-1 are greatly reduced in number. *Ldlr*$^{-/-}$ *Blimp-1*$^{fl/fl}$ *Cd23-Cre*; mice with B cells selectively deficient in Blimp-1, *Ldlr*$^{-/-}$ *Blimp-1*$^{+/+}$ *Cd23-Cre*; control mice. *P < 0.05; results are means ± SEM, n = 4-6 mice per group. Scale Bar, 100μm.

Supplementary figures

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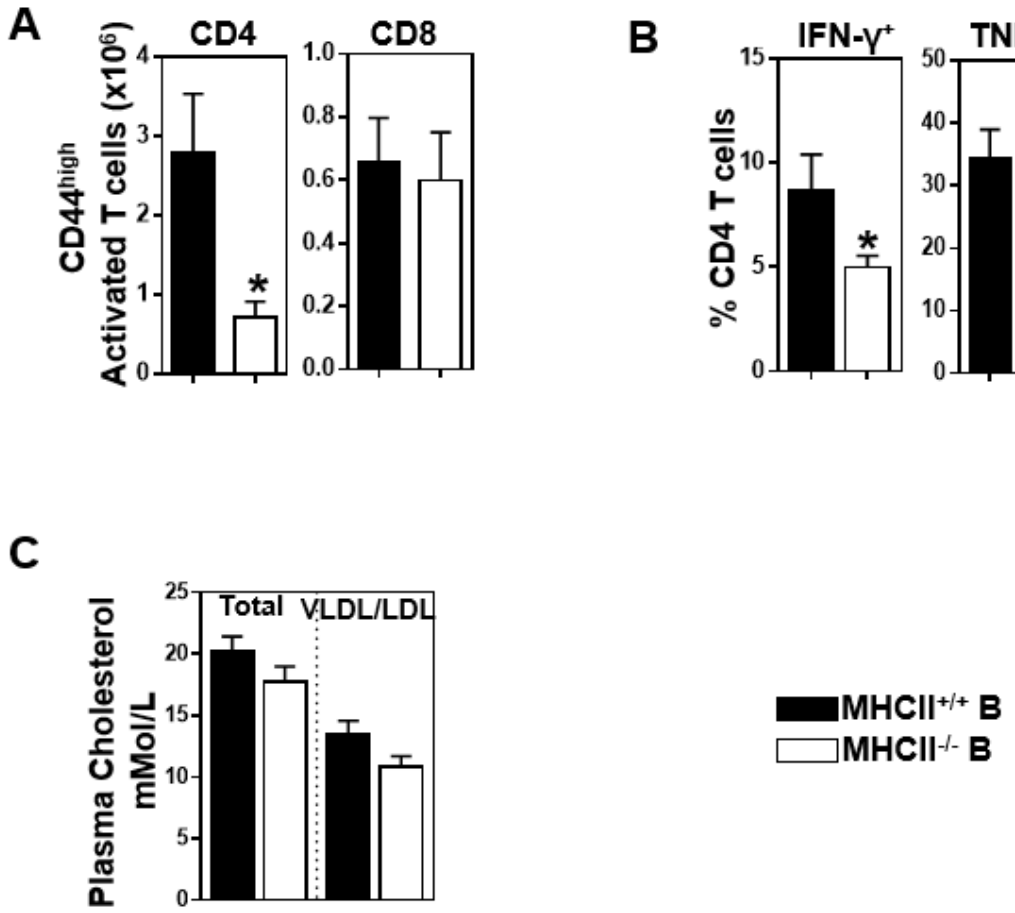
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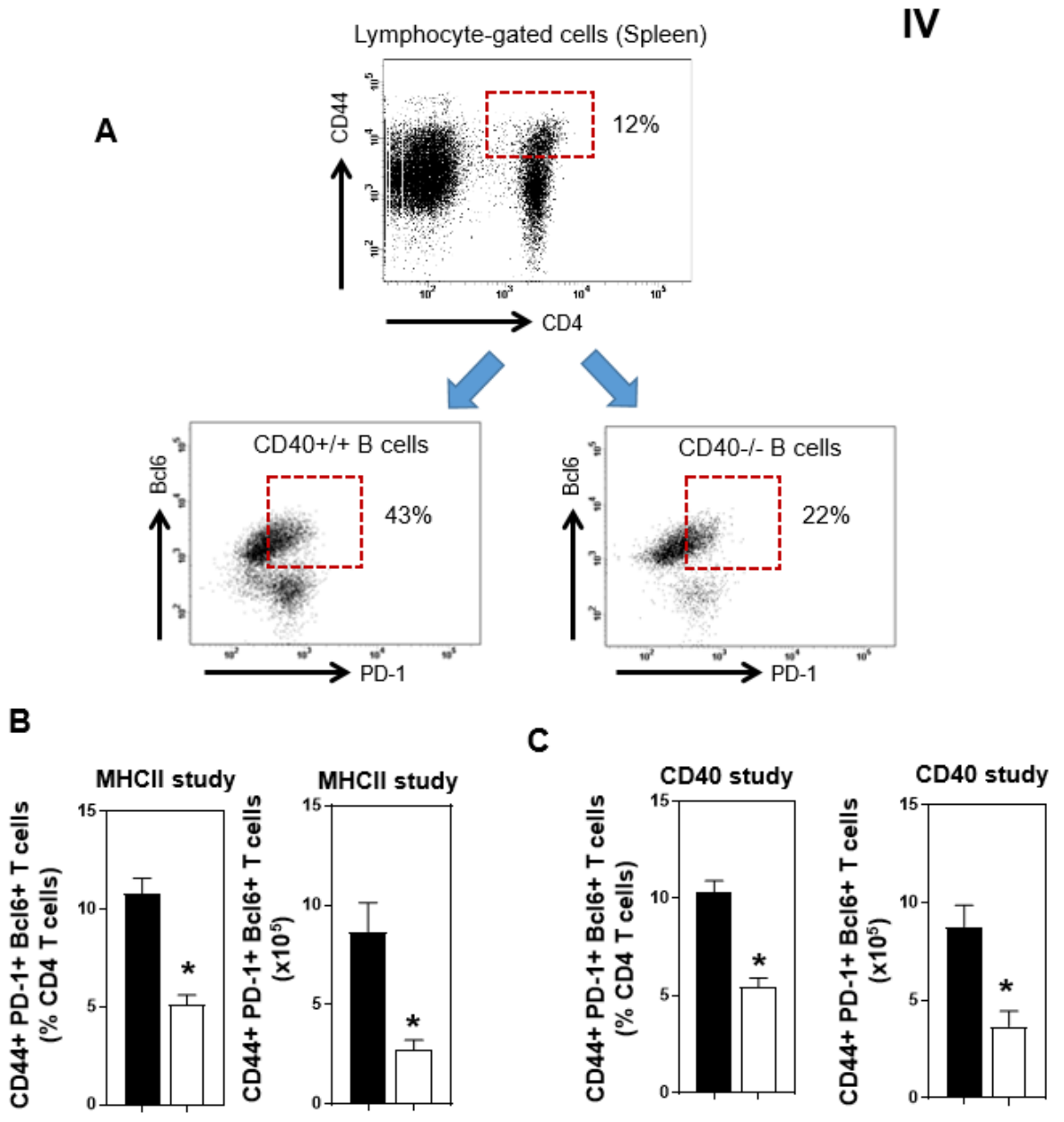
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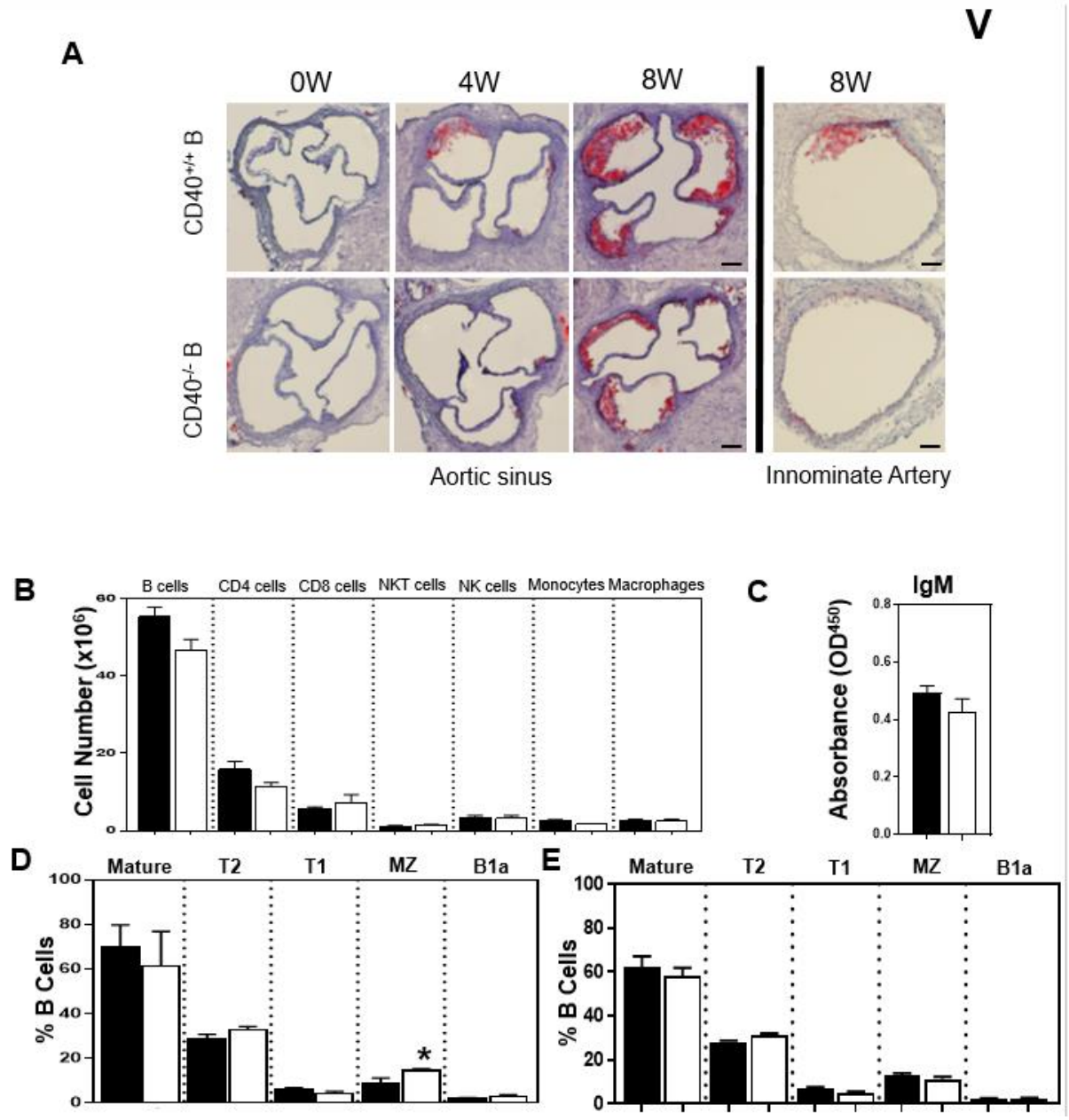
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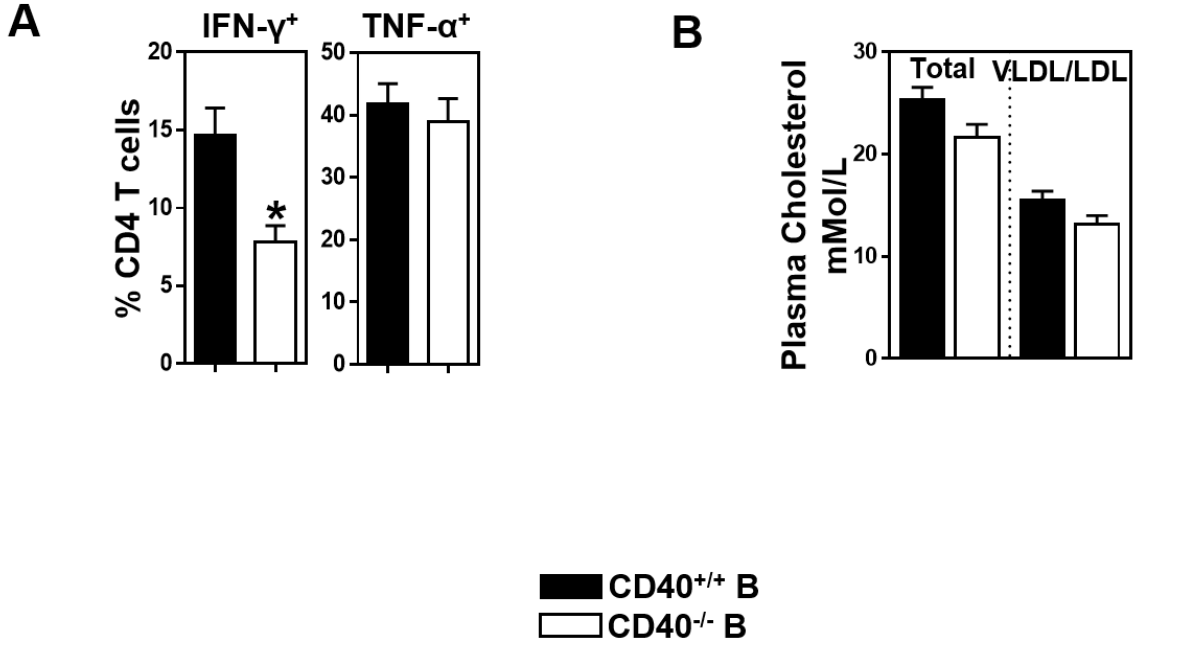
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CD40-/- B

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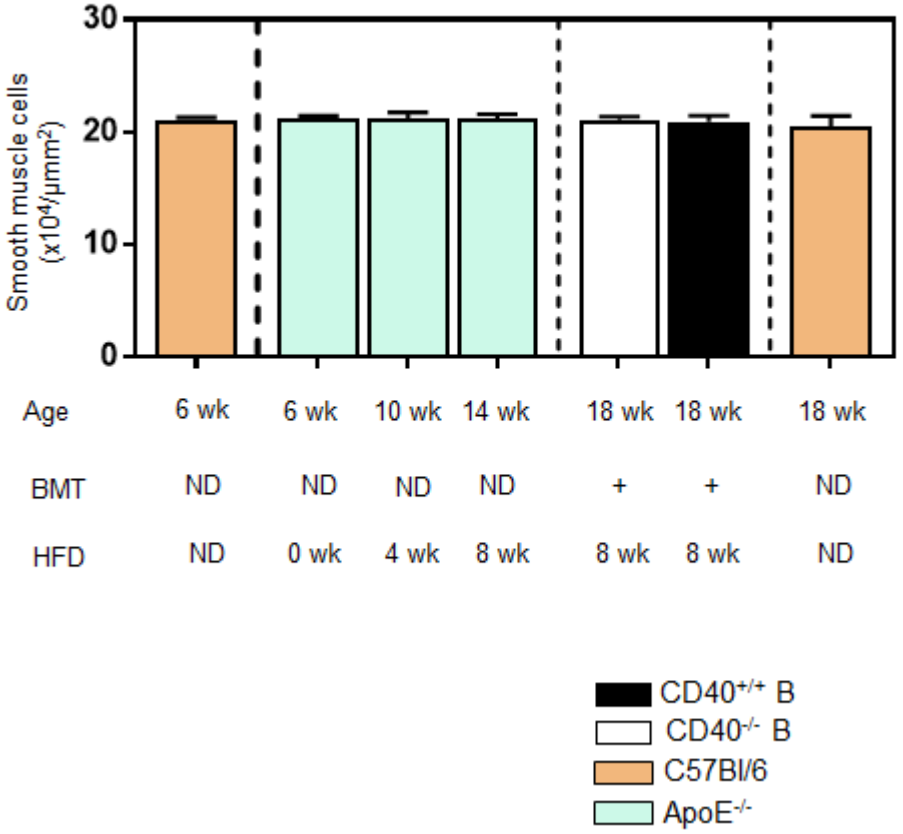
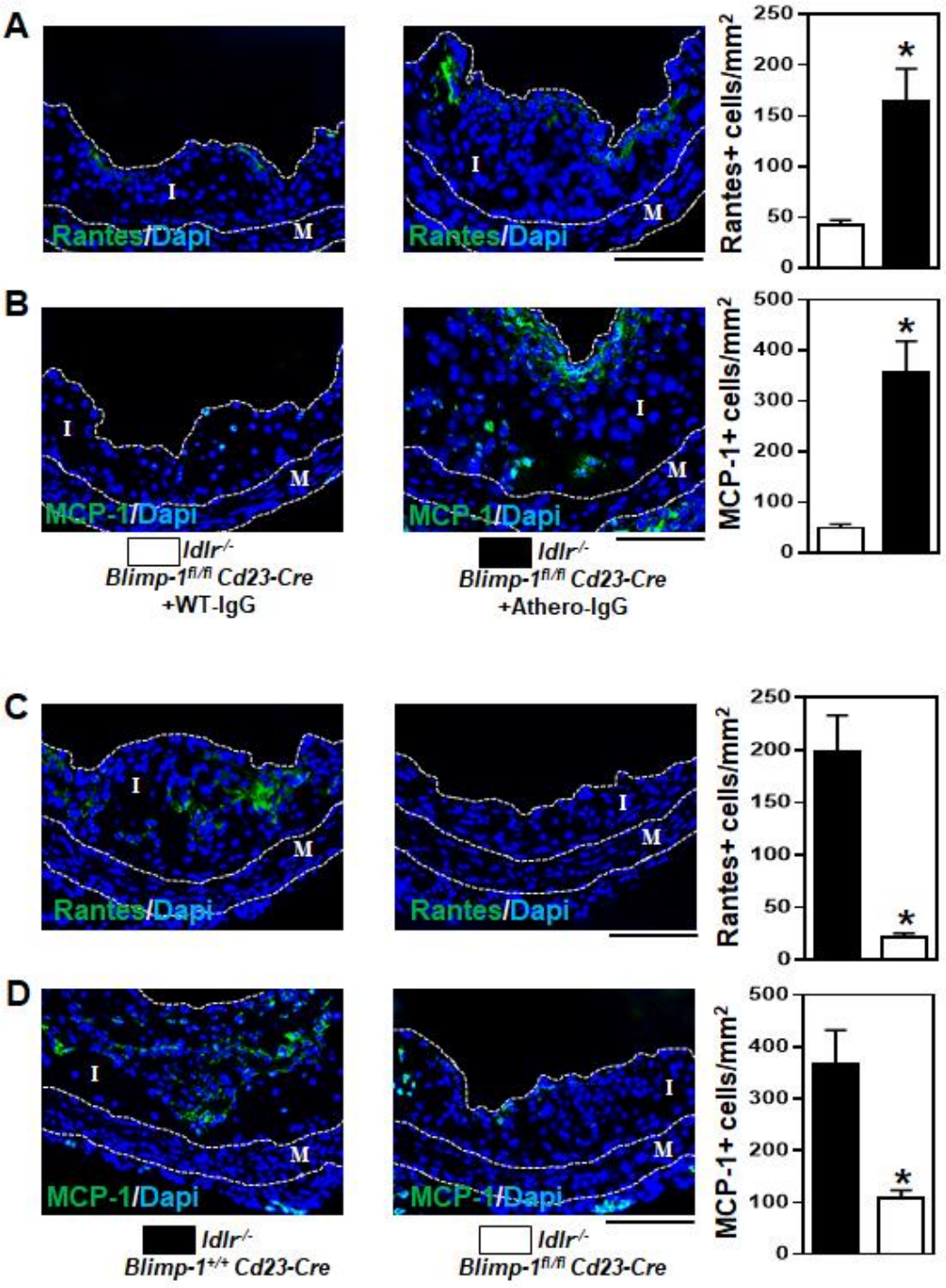
2

VII


Smooth muscle cells
(x10⁴/µmm²)
30
20
10
0
Age 6 wk 6 wk 10 wk 14 wk 18 wk 18 wk 18 wk
BMT ND ND ND ND + + ND
HFD ND 0 wk 4 wk 8 wk 8 wk 8 wk ND
CD40+/+ B
CD40-/- B
C57Bl/6
ApoE-/-


A
I
M
Rantes/Dapi
Rantes/Dapi
Rantes+ cells/mm2
250
200
150
100
50
0
*
B
MCP-1/Dapi
MCP-1/Dapi
MCP-1+ cells/mm2
500
400
300
200
100
0
*
Idlr-/-
Blimp-1fl/fl Cd23-Cre
+WT-IgG
Idlr-/-
Blimp-1fl/fl Cd23-Cre
+Athero-IgG
C
Rantes/Dapi
Rantes/Dapi
Rantes+ cells/mm2
250
200
150
100
50
0
*
D
MCP-1/Dapi
MCP-1/Dapi
MCP-1+ cells/mm2
500
400
300
200
100
0
*
Idlr-/-
Blimp-1+/+ Cd23-Cre
Idlr-/-
Blimp-1fl/fl Cd23-Cre

1