B cell depletion reduces the development of atherosclerosis in mice

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B cell depletion significantly reduces the burden of several immune-mediated diseases. However, B cell activation has been until now associated with a protection against atherosclerosis, suggesting that B cell–depleting therapies would enhance cardiovascular risk. We unexpectedly show that mature B cell depletion using a CD20-specific monoclonal antibody induces a significant reduction of atherosclerosis in various mouse models of the disease. This treatment preserves the production of natural and potentially protective anti–oxidized low-density lipoprotein (oxLDL) IgM autoantibodies over IgG type anti-oxLDL antibodies, and markedly reduces pathogenic T cell activation. B cell depletion diminished T cell–derived IFN-γ secretion and enhanced production of IL-17; neutralization of the latter abrogated CD20 antibody–mediated atheroprotection. These results challenge the current paradigm that B cell activation plays an overall protective role in atherogenesis and identify new antiatherogenic strategies based on B cell modulation.

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Abbreviations used: Apoe, apolipoprotein E; Ldlr, LDL receptor; oxLDL, oxidized low-density lipoprotein.

Atherosclerosis-related cardiovascular diseases are the leading cause of mortality worldwide. Immune-mediated reactions initiated in response to multiple potential antigens, including oxidatively modified lipoproteins and phospholipids, play prominent roles in atherosclerotic lesion development, progression, and complications (Binder et al., 2002; Hansson and Libby, 2006; Tedgui and Mallat, 2006). Besides the critical requirement for monocytes/macrophages (Smith et al., 1995), adaptive immunity substantially contributes to the perpetuation of the immunoinflammatory response, further promoting vascular inflammation and lesion development (Binder et al., 2002; Hansson and Libby, 2006; Tedgui and Mallat, 2006). Mice on a severe combined immunodeficiency or Rag-deficient background show reduced susceptibility to atherosclerosis under moderate cholesterol overload (Dansky et al., 1997; Daugherty et al., 1997; Zhou et al., 2000). Resupplementation of these mice with purified T lymphocytes accelerates lesion development (Zhou et al., 2000), even though it does not fully recapitulate lesion development of the immunocompetent mice. The proatherogenic T cells are related to the Th1 lineage (Gupta et al., 1997; Buono et al., 2005), and are counterregulated by both Th2 (Binder et al., 2004; Miller et al., 2008) and T reg cell responses (Ait-Oufella et al., 2006; Tedgui and Mallat, 2006).

The development of atherosclerosis is also associated with signs of B cell activation, particularly manifested by enhanced production of natural IgM type and adaptive IgG type anti–oxidized

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low-density lipoprotein (oxLDL) autoantibodies (Shaw et al., 2000; Caligiuri et al., 2002). However, in contrast to other immune-mediated diseases, i.e., rheumatoid arthritis and systemic lupus erythematosus, B cells have been assigned a protective role in atherosclerosis (Caligiuri et al., 2002; Major et al., 2002; Binder et al., 2004; Miller et al., 2008). Although IgG type anti-oxLDL antibodies show variable association with vascular risk, circulating levels of IgM type anti-oxLDL antibodies have been more frequently linked with reduced vascular risk in humans (Karvonen et al., 2003; Tsimikas et al., 2007). In mice, IL-5– and IL-33–mediated atheroprotective effects have been indirectly associated with specific B1 cell activation and enhanced production of natural IgM type anti-oxLDL antibodies (Binder et al., 2004; Miller et al., 2008). On the other hand, splenectomy (Caligiuri et al., 2002) or transfer of μMT-deficient (B cell–deficient) bone marrow (Major et al., 2002) into lethally irradiated atherosclerosis-susceptible mice resulted in profound reduction of IgG (Caligiuri et al., 2002) or total (Major et al., 2002) anti-oxLDL antibody production, and was associated with acceleration of lesion development. These studies led to the current paradigm that overall B cell activation is atheroprotective. Surprisingly, however, whether mature B cell depletion accelerates atherosclerotic lesion development in immunocompetent mice, as expected from previous studies, is still unexplored. This is a critical question given the potentially important risk of cardiovascular complications that might arise from the clinical use of B cell–depleting CD20-targeted immune therapy in patients with severe rheumatoid arthritis or systemic lupus erythematosus, who are at particularly high risk of cardiovascular diseases (for review see Roman et al., 2001). We have therefore designed a series of experiment to address this important question.

RESULTS AND DISCUSSION

CD20 antibody–mediated B cell depletion reduces the development of atherosclerosis both in apolipoprotein E–deficient (*Apoe*$^{-/-}$) and LDL receptor–deficient (*Ldlr*$^{-/-}$) mice

To directly assess the role of B cells in atherosclerosis, we examined lesion development in mice with or without

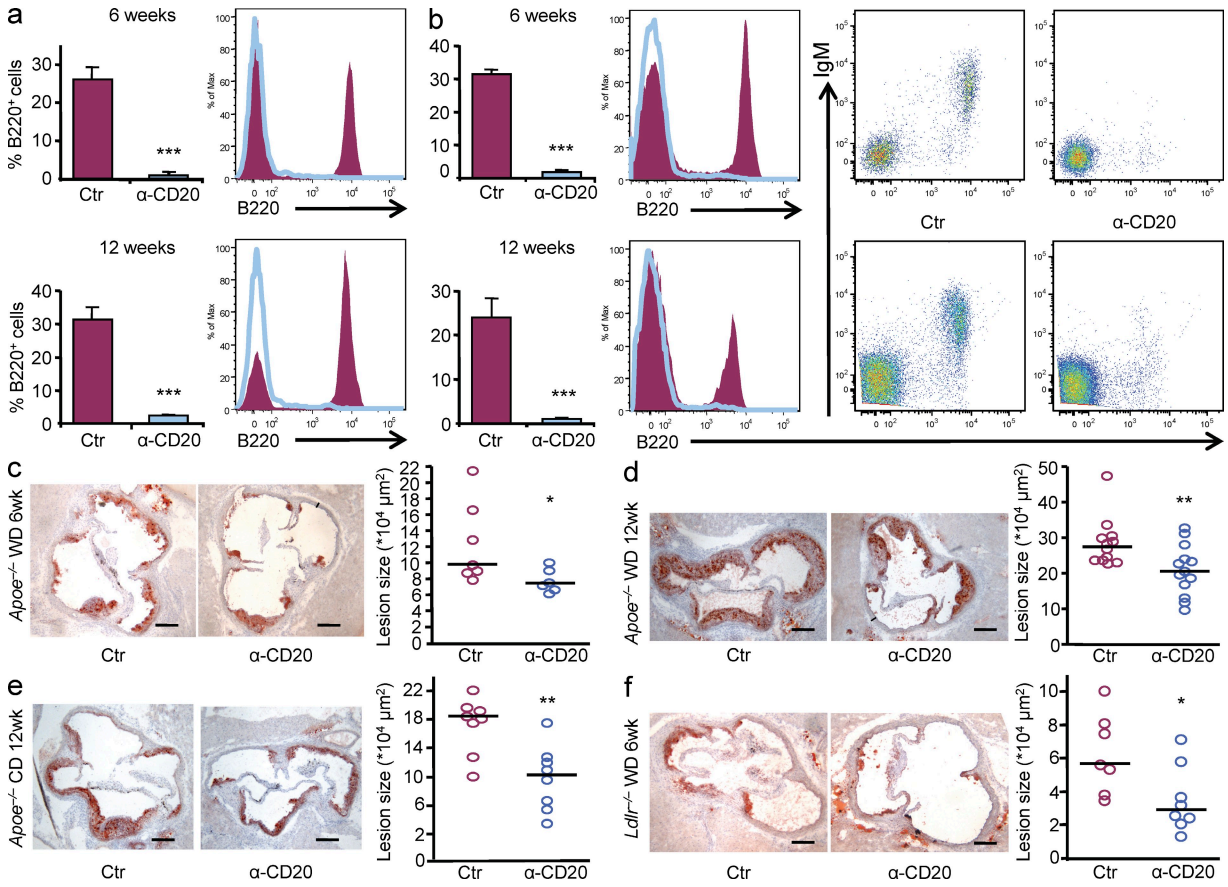

Figure 1. CD20 mAb (α-CD20) treatment depletes B cells and reduces the development of atherosclerosis. (a and b) Efficiency of B cell (B220$^+$ or IgM$^+$B220$^+$) depletion in blood (gated on lymphocytes, low forward scatter/low side scatter; a) and spleens (gated on total viable splenocytes; b) of *Apoe*$^{-/-}$ mice fed a Western diet for either 6 or 12 wk and treated with α-CD20 (blue) or a control antibody (magenta). Data are representative of four (spleen) or eight (blood) mice per group and per experiment, and two separate experiments. (c–f) Reduction of atherosclerosis development after α-CD20 therapy in four different experiments using *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice fed either a chow diet (CD) or a Western diet (WD). Representative photomicrographs of oil red O–stained aortic sinuses are shown for each experimental setting along with quantification of intimal lesion size. Horizontal bars indicate median values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bars, 200 μm.

B cell depletion. We first used *Apoe*$^{-/-}$ mice fed a high fat Western diet, a model previously shown to be associated with significant B cell activation and previously used to demonstrate the protective role of B cells in atherosclerosis (Caligiuri et al., 2002). To deplete B cells, mice were treated every 3 wk with a previously validated mouse monoclonal CD20 antibody (Uchida et al., 2004a,b) for either 6 or 12 wk. Control mice received a control mAb. As expected (Uchida et al., 2004a; Hamaguchi et al., 2005), treatment with CD20 mAb led to sustained and profound reduction of the number of mature B cells in the blood (Fig. 1 a), spleen (Fig. 1 b), peritoneum, and bone marrow (Fig. S1). B220^{high} IgM^{+} cells were severely depleted (92–100%) at all studied sites. Spleen B220^{low} IgM^{+} cells also showed a marked reduction (~80%). However, as previously observed (Uchida et al., 2004a), immature bone marrow B220^{low} IgM^{+} cells (Fig. S1) were less sensitive to CD20 mAb–mediated depletion. Treatment with CD20 mAb for 6 wk did not affect plasma cholesterol levels (6.4 ± 0.9 vs. 6.3 ± 0.8 g/liter in control and CD20 mAb–treated groups, respectively; P = 0.88) but unexpectedly led to a significant reduction, not acceleration, of atherosclerotic lesion development (Fig. 1 c). We subsequently analyzed the experiments of *Apoe*$^{-/-}$ mice treated for 12 wk under a high fat diet and still found a significant reduction of atherosclerosis at two different vascular sites (Fig. 1 d and Fig. S2) despite similar plasma cholesterol levels (18.7 ± 1.1 vs. 17.9 ± 1 g/liter in control IgG and anti-CD20–treated groups, respectively; P = 0.68). To rule out the possibility that the atheroprotective effect of CD20 mAb treatment was caused by the use of a mouse model that generates excessive inflammation in response to a very high lipid overload, we examined the effect of B cell depletion in *Apoe*$^{-/-}$ mice fed a chow diet. Treatment of these mice with CD20 antibody for 12 wk also resulted in significant reduction of lesion development (Fig. 1 e) despite similar plasma cholesterol levels (5.5 ± 0.6 vs. 5.7 ± 0.8 g/liter in control IgG and CD20 mAb–treated groups, respectively; P = 0.96). The elevated plasma cholesterol levels in *Apoe*$^{-/-}$ mice are mostly of the very low LDL subtype, whereas elevated LDL is the major atherosclerosis risk factor in humans. Thus, we examined the effects of B cell depletion in the *Ldlr*$^{-/-}$ mouse model. Again, treatment of *Lldr*$^{-/-}$ mice with CD20 mAb led to marked B cell depletion (Fig. S3) and to significant reduction of atherosclerosis (Fig. 1 f). Overall, these studies provide solid evidence for an unsuspected proatherogenic role of B cells in three mouse models of atherosclerosis.

CD20 antibody–mediated B cell depletion preserves the production of natural anti-oxLDL IgM antibodies over IgG type anti-oxLDL antibodies

We next addressed the potential mechanisms responsible for atheroprotection after B cell depletion. We found that treatment with CD20 depleting antibody resulted in a profound reduction of IgG type anti-oxLDL antibodies both at 6 and 12 wk of treatment (Fig. 2), which was consistent with the profound depletion of B220^{high} cells in blood, spleen, and bone marrow (Fig. 1 and Fig. S1). Reduction of anti-oxLDL

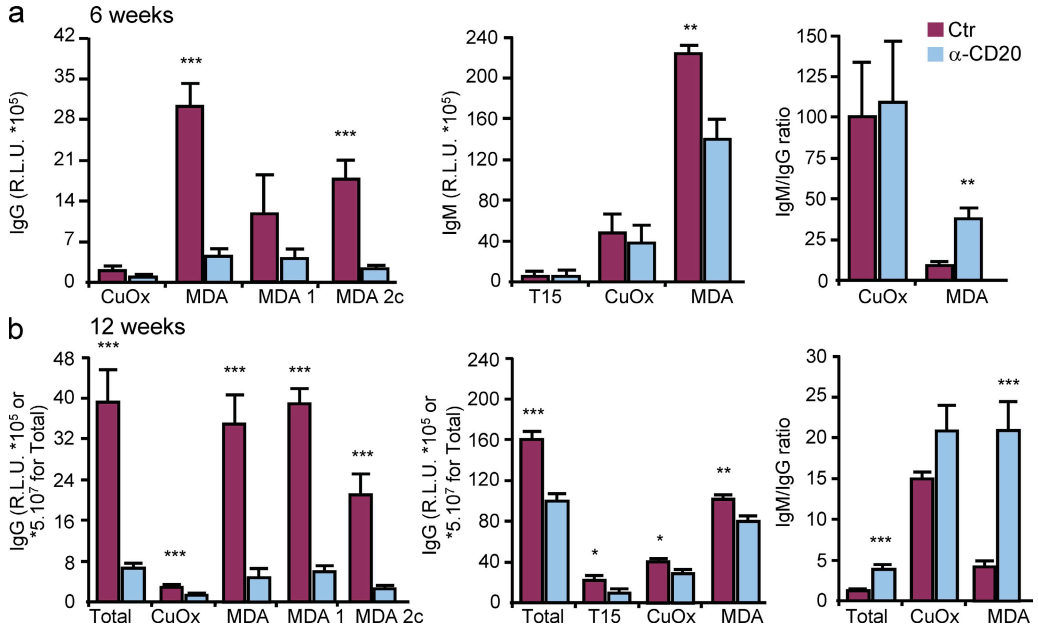

Figure 2. CD20 mAb (α-CD20) reduces the production of anti-oxLDL antibodies. (a and b) Antibody (IgG and IgM) responses to copper-oxidized (CuOx) or malondialdehyde (MDA)-modified LDLs in the circulating blood of *Apoe*$^{-/-}$ mice fed a Western diet for either 6 (a) or 12 wk (b) and treated with α-CD20 or a control antibody. MDA 1 indicates IgG1 type antibodies against MDA-LDL, and MDA 2c indicates IgG2c type antibodies. Data are representative of 6 (6 wk), 12 (Ctr; 12 wk), and 13 (α-CD20; 12 wk) mice per group and two different experiments. Mean values ± SEM are represented. *, P < 0.05; **, P < 0.01; ***, P < 0.001. R.L.U., relative light units.

IgG antibodies might have limited the potentially deleterious consequences of immune complex formation on atherosclerosis (Hernández-Vargas et al., 2006). However, in other studies and particularly after splenectomy, profound reduction in anti-oxLDL IgG levels was observed in association with acceleration, not reduction, of atherosclerosis (Caligiuri et al., 2002). Thus, in the absence of studies directly addressing the role of IgG type anti-oxLDL antibodies on atherosclerosis, changes in anti-oxLDL IgG levels after CD20 mAb treatment could not be held responsible for lesion reduction. Levels of IgM type antibodies against either copper-oxidized or malondialdehyde-modified LDL were also reduced after 6 or 12 wk of CD20-targeted therapy (Fig. 2). IgM type antibodies are endowed with atheroprotective properties (Lewis et al., 2009), and their reduction after CD20 mAb therapy could not account for atheroprotection. It is interesting to note, however, that IgM type anti-oxLDL and T15id$^+$ IgM antibodies showed a much lower reduction compared with IgG type antibodies (Fig. 2), which is consistent with the lower efficiency of CD20 mAb on peritoneal B1 cell depletion (Fig. S4; Hamaguchi et al., 2005). This might have preserved an atheroprotective pathway. IgM type antibodies dominate the humoral response to oxLDL in *Apoe*$^{-/-}$ mice (Palinski et al., 1994, 1996) and are increased even at a young age (before the initiation of CD20 mAb treatment in this study), which may also explain, at least in part, the persistence of a significant IgM level after CD20 immunotherapy, a treatment that does not dramatically affect preexisting antibody titers (DiLillo et al., 2008).

CD20 antibody–mediated B cell depletion reduces DC and T cell activation, and inhibits T cell infiltration within atherosclerotic lesions

We next examined atherosclerotic lesion composition to gain more insight into the mechanisms of atheroprotection. In addition to the significant reduction of macrophage accumulation (Fig. S5), CD20 mAb treatment was associated with a marked reduction of T lymphocyte accumulation within the lesions (Fig. 3 a), suggesting a role for B cells in driving T cell–dependent lesion inflammation. As expected at this stage of lesion formation, very few B cells were detected within the plaques or within the adventitial layer (Fig. S6), suggesting that modulation of lesion T cell accumulation by CD20 mAb therapy most likely occurred as a consequence

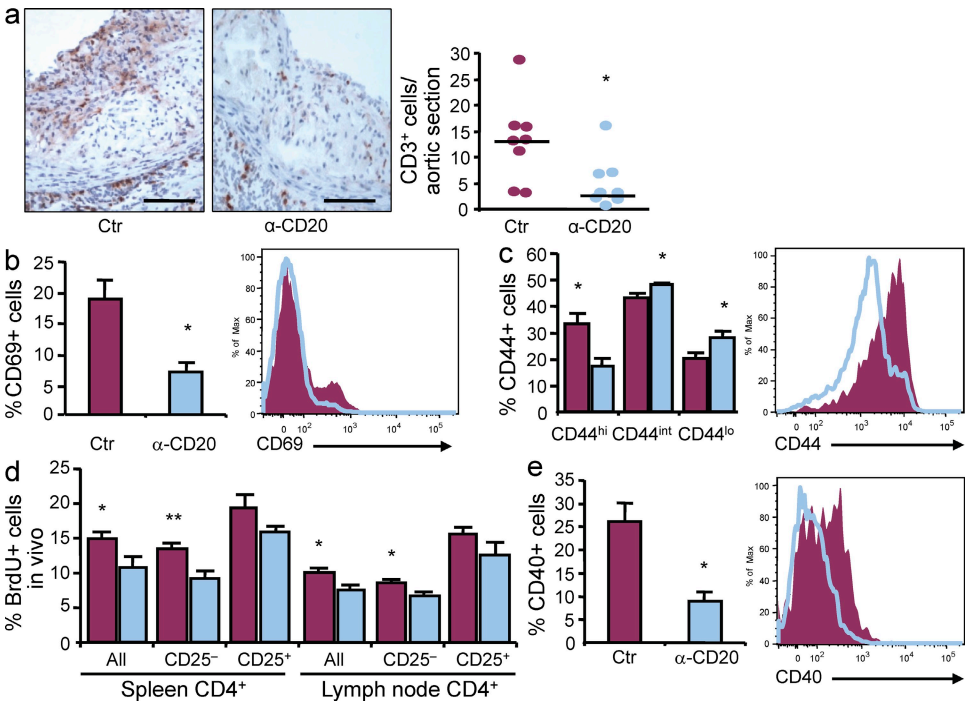

Figure 3. CD20 mAb (α-CD20) reduces systemic T cell activation and limits T cell accumulation within atherosclerotic lesions. (a) Representative photomicrographs and quantitative analysis of CD3 staining in atherosclerotic lesions of *Apoe*$^{-/-}$ mice fed a Western diet for 12 wk and treated with CD20 mAb (α-CD20) or control antibody (Ctr). Horizontal bars indicate median values. Data are representative of one experiment with eight mice per group. Bars, 100 µm. (b and c) Representative histograms of CD69 and CD44 expression on gated CD4$^+$ spleen cells of *Apoe*$^{-/-}$ mice fed a Western diet for 12 wk and treated with CD20 mAb (α-CD20; blue) or control antibody (Ctr; magenta). CD44 expression was divided into low, intermediate, or high. Data are representative of four mice per group and two separate experiments. (d) Percentage of BrdU-positive cells determined using flow cytometry among spleen- or subcutaneous lymph node–derived CD4$^+$ populations. BrdU was administered the week before animal sacrifice (after 6 wk of a Western diet), as described in Materials and methods. Data are representative of three mice per group and two separate experiments. (e) Representative histograms of CD40 expression on gated CD11c$^+$ spleen cells of *Apoe*$^{-/-}$ mice fed a Western diet for 12 wk and treated with CD20 mAb (α-CD20; blue) or control antibody (Ctr; magenta). Data are representative of four mice per group and two separate experiments. Mean values ± SEM are represented. *, $P < 0.05$; **, $P < 0.01$.

of systemic modulation of T cell function. To address this hypothesis, we examined T cell activation and proliferation. Interestingly, we consistently found marked reductions in CD69 and CD44^{high} expression on spleen-derived CD4$^+$ T cells of mice treated with CD20 antibody compared with controls at both 6 wk (not depicted) and 12 wk (Fig. 3, b and c) of a high fat diet, indicating reduced T cell activation. B cell depletion also led to significant reduction of in vivo BrdU staining of effector CD4$^+$CD25$^-$ T cells, suggesting reduced proliferation (Fig. 3 d). This is consistent with the lower proliferative potential of CD4$^+$ cells recovered from CD20-treated mice and co-cultured with purified CD11c$^+$ cells in the presence of CD3 stimulation (Fig. S7 a). In addition, replacement of control CD11c$^+$ cells with DCs isolated from CD20-depleted mice led to a reduction of (control) T cell proliferation in vitro (Fig. S7 a). This is also consistent with the marked reduction of CD40 expression on CD11c$^+$ DCs recovered from CD20 mAb–treated mice (Fig. 3 e). Moreover, we show that incubation of DCs with activated B cells leads to enhanced CD40, CD80, and MHC-II expression on DCs (Fig. S7 b). Thus, a major consequence of B cell depletion using CD20 antibody is a marked reduction of DC and T cell activation in vivo, which could potentially account for its atheroprotective effect.

CD20 antibody–mediated B cell depletion switches the immune response toward diminished IFN-γ secretion and enhanced production of IL-17

T cell–derived cytokines significantly alter lesion development (Tedgui and Mallat, 2006). Therefore, we examined the consequences of B cell depletion on cytokine production by purified T cells. We found a marked reduction of proatherogenic IFN-γ by purified T cells recovered from CD20 mAb–treated mice compared with controls (Fig. 4 a). Of note, this was associated with a deviation of the immune response toward a significant increase of T cell–derived (Fig. 4, b and c) and circulating IL-17A production in CD20 mAb–treated animals compared with controls (26 ± 4 vs. 0 pg/ml, respectively). Replacement of control CD11c$^+$ cells with DCs isolated from CD20-depleted mice significantly shifted (control) T cell cytokine production toward a higher IL-17/IFN-γ ratio (Fig. S7 a), indicating a contribution of DCs to the observed cytokine switch. Recent studies in our laboratory identified an unexpected regulatory and protective role for IL-17A in atherosclerosis under a context of reduced IFN-γ production (Taleb et al., 2009). In addition, IL-17A has been shown to modulate Th1 polarization (O'Connor et al., 2009). Thus, the T cell cytokine profile induced by B cell depletion is compatible with the observed atheroprotective effects.

IL-17A neutralization abrogates CD20 antibody–mediated atheroprotection

To examine whether CD20 mAb–induced changes in T cell cytokine profile (reduced IFN-γ and increased IL-17) could be responsible for CD20 mAb–dependent atheroprotection, CD20 mAb was administered to *Apoe*$^{-/-}$ mice (on a high fat diet for 6 wk) in the presence of control or anti–IL-17A neutralizing antibody. IL-17 neutralization led to increased IFN-γ production in the spleen (not depicted) and atherosclerotic aortas, and completely abrogated the atheroprotective effects of CD20 mAb therapy (Fig. 5, a and b) despite similar circulating cholesterol levels, similar efficiency of B cell depletion (Fig. S8), and no significant changes in anti-oxLDL antibodies levels (Fig. S8). We reproduced these results in a second separate experiment (Fig. 5 c). Collectively, these results identify a hitherto unsuspected role for B cells in driving the development of atherosclerosis through modulation of T cell activation and cytokine production.

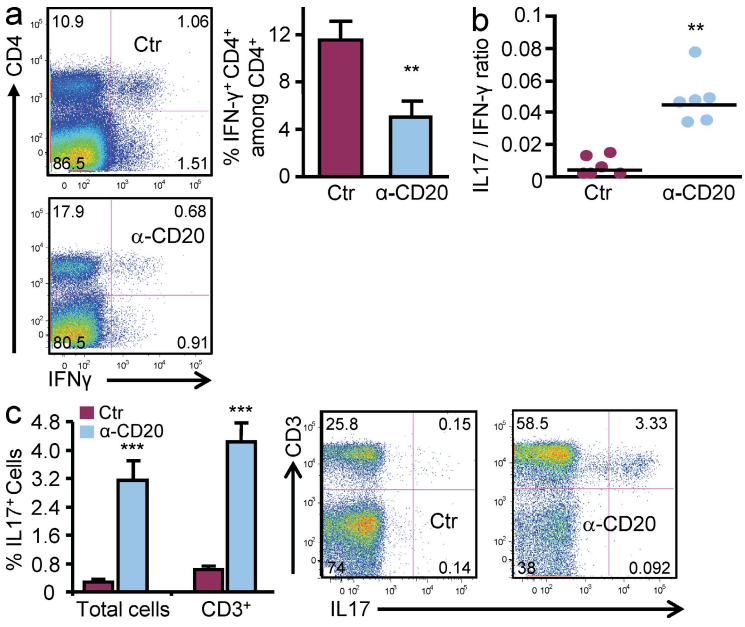

Figure 4. Treatment with CD20 mAb (α-CD20) induces a switch toward lower IFN-γ but higher IL-17 production. (a) Representative examples and quantitative analysis of intracellular IFN-γ staining of isolated splenocytes from *Apoe*$^{-/-}$ mice fed a Western diet for 6 wk and treated with CD20 mAb (α-CD20; blue) or control antibody (Ctr; magenta). Plots are gated on CD4$^+$ cells. Numbers in each quadrant indicate percentages of cells. Histograms depict mean values ± SEM. Similar results were obtained after 12 wk of a high fat diet (not depicted). (b) Quantitative analysis of IL-17 and IFN-γ production (ratio of Il-17/IFN-γ is depicted) in supernatants of purified spleen-derived T cells (*Apoe*$^{-/-}$ mice fed a Western diet for 12 wk) after stimulation with anti-CD3 antibody in the presence of purified CD11c$^+$ cells. Horizontal bars indicate median values. Data are representative of four to five mice per group and two separate experiments. (c) Representative examples and quantitative analysis of intracellular IL-17 staining among spleen CD3$^+$ cells, determined using flow cytometry on isolated splenocytes from *Ldlr*$^{-/-}$ mice fed a Western diet for 6 wk and treated with CD20 mAb (α-CD20; blue) or control antibody (Ctr; magenta). Mean values ± SEM are represented. Data are representative of five mice per group and one experiment. **, $P < 0.01$; ***, $P < 0.001$.

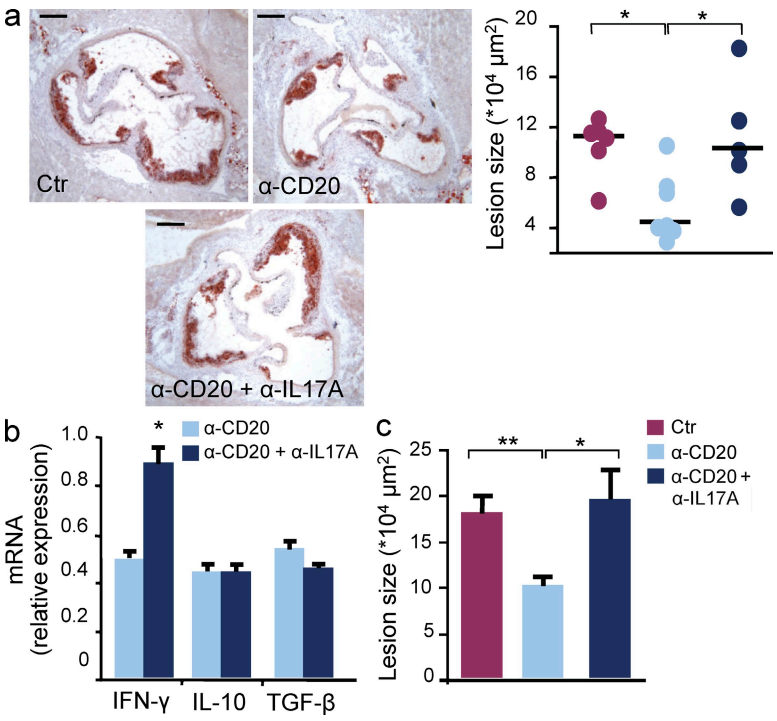

Figure 5. IL-17A neutralization abrogates CD20 antibody–mediated atheroprotection. (a) Representative photomicrographs of oil red O–stained aortic sinuses and quantitative analysis of intimal lesion size in *Apoe*$^{-/-}$ mice fed a Western diet for 6 wk and treated with control antibodies for both CD20 and IL-17 (Ctr group), CD20 mAb and a control antibody for IL-17 (α-CD20 group), or neutralizing anti–L-17A mAb and a CD20 mAb (α-CD20 + α–IL-17A group). Horizontal bars indicate median values. *, $P < 0.05$. Bars, 200 µm. This experiment was repeated in c. (b) Quantitative analysis of IFN-γ, IL-10, and TGF-β mRNA expression (relative to GAPDH) in the thoracic aortas of *Apoe*$^{-/-}$ mice fed a Western diet for 6 wk and treated with CD20 mAb or CD20 mAb and anti–L-17A mAb. Mean values ± SEM are representative of five to seven mice per group and one experiment. *, $P < 0.05$. (c) Quantitative analysis of intimal lesion size in an additional set of *Apoe*$^{-/-}$ mice fed a Western diet for 6 wk and treated with control antibodies for both CD20 and IL-17 (Ctr group; $n = 8$), CD20 mAb and a control antibody for IL-17 (α-CD20 group; $n = 7$), or neutralizing anti–L-17A mAb and a CD20 mAb (α-CD20 + α–IL-17A group; $n = 9$). Data represent mean values ± SEM. *, $P < 0.05$; **, $P < 0.01$.

Our results may seem in disagreement with previous studies showing that both µMT deficiency (Major et al., 2002) and splenectomy (Caligiuri et al., 2002) accelerate atherosclerosis in mice. However, these studies did not directly address the role of mature B cell depletion on atherosclerosis in immunocompetent mice. Several other concomitant immune cell dysfunctions (Moulin et al., 2000; Ngo et al., 2001; João et al., 2004; AbuAttieh et al., 2007) might have contributed to enhanced lesion development in µMT-deficient animals. Furthermore, the reported limitation of atherosclerosis acceleration in splenectomized mice after reconstitution with purified B cells could have been confounded by the reduction of plasma cholesterol levels in B cell–reconstituted mice (Caligiuri et al., 2002), and could not be selectively attributed to B cells because T cell reconstitution also resulted in atheroprotection (Caligiuri et al., 2002). Finally, it should be noted that although B cell depletion significantly limited lesion development in our present studies, the roles of specific subtypes of B cells in driving or controlling atherosclerosis merit further investigation. More particularly, it will be important to address the respective roles of regulatory (Yanaba et al., 2008) versus nonregulatory B cells in these processes (Bouaziz et al., 2007). Finally, it could be argued that skewing T cell responses from Th1 to IL-17 production in the absence of mature B cells would not be helpful in other autoimmune/inflammatory diseases known to be mediated in part by Th17 cells. However, recent studies have provided data indicating that not all Th17 cells are endowed with the same pathogenic potential (McGeachy et al., 2007). These issues merit further investigation.

In conclusion, we provide strong evidence that mature B cell depletion reduces the development of atherosclerosis in mice. Our results challenge the paradigm that overall B cell function is atheroprotective and show that a major B cell role in atherosclerosis is to drive T cell activation toward enhanced proatherogenic Th1 immune response and limited production of atheroprotective IL-17 (Fig. S9). Although limited vascular B cell infiltration is detectable in the early stages of atherosclerosis, B cell accumulation substantially increases with time. It localizes within and around advanced atherosclerotic coronary lesions and atherosclerotic abdominal aortic aneurysms (Moos et al., 2005) of mice and humans, and is even prominent in vascular inflammation associated with other immune-mediated diseases (Aubry et al., 2004). Inhibition of excessive B cell activation either through depletion or immune modulation might substantially limit vascular inflammation and atherosclerotic lesion development.

MATERIALS AND METHODS

Animals. All mice were on a C57BL/6 background. *Apoe*$^{-/-}$ mice were 10-wk-old males maintained on a chow diet for 12 wk or put on a Western diet (20% fat, 0.15% cholesterol, 0% cholate) for either 6 or 12 wk. *Ldlr*$^{-/-}$ mice were 10-wk-old males put on a Western diet for either 6 or 12 wk. At 10 wk old, mice were treated i.p. with a previously validated mouse CD20 mAb (Uchida et al., 2004a,b) or control IgG (200 µg every 3 wk) for either 6 or 12 wk. In some experiments, mice received an i.p. injection of either purified neutralizing anti–IL-17A–specific antibody (200 µg/mouse, twice per week; Uyttenhove and Van Snick, 2006; Uyttenhove et al., 2007; Taleb et al., 2009) or control IgG for 6 wk. Experiments were conducted according to the French veterinary guidelines and those formulated by the European Community for experimental animal use (L358-86/609EEC), and were approved by the Institut National de la Santé et de la Recherche Médicale.

Extent and composition of atherosclerotic lesions. Quantification of lesion size and composition was performed as previously described (Taleb et al., 2007).

Cell recovery and purification, culture, proliferation, and cytokine assays. $CD11c^+$ and $CD4^+$ cells were purified and processed for cell proliferation assays and cytokine production as previously described in detail (Taleb et al., 2007). For cell proliferation experiments, $CD4^+$ cells were cultured in round-bottom 96-well microplates. Cells were stimulated with 1 μg/ml of purified soluble CD3-specific antibody (BD) in the presence of 10^4 antigen-presenting cells purified on CD11c-coated magnetic beads (Miltenyi Biotec). For cytokine measurements, $CD4^+$ T cells were cultured at 10^5 cells/well for 48 h on anti-CD3–coated microplates (5 μg/ml) in the presence of 2×10^4 $CD11c^+$ cells. IL-17 and IFN-γ production in the supernatants was measured using specific ELISAs (BD and R&D Systems).

Flow cytometry. Allophycocyanin (APC)-conjugated anti-CD3ε (145-2C11), FITC- or PE-Cy7–conjugated anti-CD4 (RM4-5), APC-conjugated anti-CD25 (PC61.5), PE-conjugated anti-CD69 (H1.2F3), APC-conjugated anti-IgM (II/41), FITC-conjugated anti-CD86 (GL1), PE-conjugated anti-CD80 (16-10A1), APC-conjugated anti-CD40 (1C10), PE-Cy7–conjugated anti-CD11c (N418), PE-Cy7–conjugated anti-CD11b (M1/70), and PE-conjugated anti-CD45R (B220; RA3-6B2) were from eBioscience. FITC-conjugated anti-CD5 (53-7.3), biotin-conjugated anti-CD44 followed by APC-conjugated streptavidin, APC-Cy7–conjugated anti-CD45R (B220; RA3-6B2), APC-conjugated anti–IFN-γ (XMG1.2), and PE-conjugated anti–IL-17A (TC11-18H10) were from BD. For blood staining, erythrocytes were lysed using FACS lysing solution (BD). For intracellular cytokine staining, lymphocytes were stimulated in vitro with leukocyte activation cocktail (BD) according to the manufacturer's instructions for 4 h. Surface staining was performed before permeabilization using an intracellular staining kit (eBioscience). For staining on aortic cells, the aorta was removed and flushed intensively with 2 mM PBS-EDTA. The aorta was cut into pieces and digested using a cocktail of enzymes for 30 min at 37°C (450 U/ml collagenase I, 375 U/ml collagenase XI, 180 U/ml hyaluronidase I, and 180 U/ml DNase I; all from Sigma-Aldrich). Forward scatter and side scatter were used to gate live cells excluding red blood cells, debris, and cell aggregates in total splenocyte, lymph node, bone marrow, aorta, and peritoneum populations. Cells were analyzed using a flow cytometer (FACSCanto II or LSR II; BD).

BrdU labeling and cell analysis. BrdU labeling was performed as previously described (Fisson et al., 2003). Mini osmotic pumps (ALZET1007D; Charles River), delivering 1.2 mg/day BrdU (Sigma-Aldrich) for 7 d, were transplanted to mice subcutaneously under isoflurane anesthesia 1 wk before sacrifice. Lymph node cells and splenocytes were stained with PE-Cy7–conjugated anti-CD4 (RM4-5) and APC-conjugated anti-CD25 (PC61.5) antibodies. BrdU detection was performed using an FITC BrdU flow kit (BD) according to the manufacturer's instructions. Cells were analyzed using a FACSCanto II or LSR II flow cytometer.

B cell–DC co-culture. Bone marrow–derived DCs were cultured from whole bone marrow using 20 ng/ml of GM-CSF (Miltenyi Biotec)–conditioned medium for 10 d. DCs were plated in 24 wells at 4×10^5 cells/well. B cells were isolated from C57BL/6 spleens using a B cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. B cells were incubated overnight in RPMI 1640 with 10 μg/ml anti–mouse IgM (Jackson ImmunoResearch Laboratories, Inc.) and 2.5 μg/ml anti–mouse CD40 (clone HM40-3; BioLegend). After washing, B cells were added on DC cultures at 2×10^6 cells per well and co-cultured for 48 h. DCs were collected for FACS analysis.

Quantitative real-time PCR. Quantitative real-time PCR was performed on an ABI Prism 7700 (Applied Biosystems) in triplicate. Cycle threshold for GAPDH (primers: GAPDH R, 5′-CGTCCCGTAGACAAAATGGTGAA-3′; GAPDH L, 5′-GCCGTGAGTGGAGTCATACTGGAACA-3′) was used to normalize gene expression. The following primer sequences were used: IL-10 (IL-10 R, 5′-GGCGCTGTCATCGATTTCT-3′; IL-10 L, 5′-ATTCATTCATGGCCTTGTAGACAC-3′), TGF-β (TGF-β R, 5′-AGCAACAATTCCTGGCGTTACCTT-3′; TGF-β L, 5′-TGGGCTCGTGGATCCACTTC-3′), and IFN-γ (IFN-γ R, 5′-GCCACGGCACAGTCATTGAAA-3′; IFN-γ L, 5′-TCGCCTTGCTGTTGCTGAAGA-3′).

Determination of circulating antibodies. Specific antibody titers to given antigens in plasma were determined by chemiluminescent ELISA, as previously described (Friguet et al., 1985; Binder et al., 2003; Chou et al., 2009).

Statistical analysis. Values are expressed as means ± SEM. Differences between values were examined using nonparametric Mann-Whitney or Kruskal-Wallis tests, and were considered significant at $P < 0.05$.

Online supplemental material. Fig. S1 shows CD20 mAb–induced B cell depletion in bone marrow and peritoneum. Fig. S2 shows that CD20 mAb treatment reduces the development of atherosclerosis in the thoracic aorta. Fig. S3 shows the efficiency of B cell depletion in *Ldlr*$^{-/-}$ mice. Fig. S4 shows the relative efficiency of CD20 mAb treatment on peritoneal B2, B1a, and B1b cell depletion in *Apoe*$^{-/-}$ mice. Fig. S5 shows that CD20 mAb treatment reduces macrophage infiltration within atherosclerotic lesions. Fig. S6 shows that very few B cells are detected with atherosclerotic arteries of *Apoe*$^{-/-}$ mice fed a Western diet for 12 wk. Fig. S7 shows modulation of DC and T cell functions through B cell activation or depletion. Fig. S8 shows that IL-17A neutralization does not affect plasma cholesterol levels, efficiency of B cell depletion, or production of anti-oxLDL antibodies. Fig. S9 shows the proposed mechanisms for the atheroprotective effect of CD20 mAb. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100155/DC1.

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