B1a B Lymphocytes Are Atheroprotective by Secreting Natural IgM That Increases IgM Deposits and Reduces Necrotic Cores in Atherosclerotic Lesions

Tin Kyaw,* Christopher Tay,* Surendran Krishnamurthi,* Peter Kanellakis, Alex Agrotis, Peter Tipping,† Alex Bobik,† Ban-Hock Toh†

Rationale: Aggravated atherosclerosis in B lymphocyte-deficient chimeric mice and reduced atherosclerosis after transfer of unfractionated spleen B lymphocytes into splenectomized mice have led to the widely held notion that B lymphocytes are atheroprotective. However, B lymphocytes can be pathogenic, because their depletion by anti-CD20 antibody ameliorated atherosclerosis, and transfer of B2 lymphocytes aggravated atherosclerosis. These observations raise the question of the identity of the atheroprotective B-lymphocyte population.

Objective: The purpose of the study was to identify an atheroprotective B-lymphocyte subset and mechanisms by which they confer atheroprotection.

Methods and Results: Splenectomy of apolipoprotein E–deficient mice selectively reduced peritoneal B1a lymphocytes, plasma IgM, and oxidized low-density lipoprotein IgM levels and lesion IgM deposits. These reductions were accompanied by increased oil red O–stained atherosclerotic lesions and increased necrotic cores, oxidized low-density lipoproteins, and apoptotic cells in lesions. Plasma lipids, body weight, collagen, and smooth muscle content were unaffected. Transfer of B1a lymphocytes into splenectomized mice increased peritoneal B1a lymphocytes; restored plasma IgM, oxidized low-density lipoprotein IgM levels, and lesion IgM deposits; and potently attenuated atherosclerotic lesions, with reduced lesion necrotic cores, oxidized low-density lipoprotein, and apoptotic cells. In contrast, transfer of B1a lymphocytes that cannot secrete IgM failed to protect against atherosclerosis development in splenectomized mice despite reconstitution in the peritoneum.

Conclusions: B1a lymphocytes are an atheroprotective B-lymphocyte population. Our data suggest that natural IgM secreted by these lymphocytes offers protection by depositing IgM in atherosclerotic lesions, which reduces the necrotic cores of lesions. (Circ Res. 2011;109:830-840.)

Key Words: B-lymphocytes ■ apoptosis ■ inflammation ■ immunoglobulin M ■ splenectomy

Atherosclerosis is an inflammatory disease of arteries initiated by lipid entry into the arterial intima. Despite introduction of lipid-lowering statins, it remains the major cause of cardiovascular deaths related to heart attacks and strokes. There is therefore a pressing need to precisely understand the role of leukocytes in the inflammatory infiltrate in atherosclerosis that can be exploited to develop novel antiinflammatory therapies.

The presence of B lymphocytes in the inflammatory infiltrate in humans and in mice has led to a search for their role in atherosclerosis. Earlier studies led to a widely held notion that B lymphocytes are protective. This conclusion is based primarily on 2 principal reports. The first was that adoptive transfer of large numbers of unfractionated spleen B lymphocytes reduced splenectomy-aggravated atherosclerosis. The next was the report of aggravated atherosclerosis in low-density lipoprotein receptor–deficient (LDL-R−/−) mice rendered B lymphocyte deficient by bone marrow transplantation from mice deficient in the μ-chain of immunoglobulin into irradiated recipients. However, our recent observation that of Ait-Oufella et al that depletion of B lymphocytes by anti-CD20 antibody ameliorated atherosclerosis indicate that B lymphocytes can be proatherogenic. Furthermore, we identified conventional B2 lymphocytes as a proatherogenic B-lymphocyte population, because transfer of this population to lymphocyte-deficient or B lymphocyte–
deficient apolipoprotein E–deficient (ApoE<sup>−/−</sup>) mice aggravated atherosclerosis. These observations raise the question of the identity of the protective B-lymphocyte population in atherosclerosis.

In a search for an atheroprotective B-lymphocyte subset, we were guided by reports that splenectomy is associated with augmented atherosclerosis in humans and in experimental animals. In a 19-year follow-up of World War II veterans with trauma-induced splenectomy, a high incidence of acute myocardial infarction was reported compared with those without splenectomy. In animal studies, splenectomized mice, rats, and rabbits showed significantly increased atherosclerotic lesions compared with sham-operated controls.

The report that B1a lymphocytes are depleted in splenectomized or asplenic mice suggests that the aggravated atherosclerosis in splenectomized mice may be the consequence of the loss of peritoneal B1a lymphocytes. Because B1a lymphocytes produce the majority of natural IgM, and IgM has been shown to protect against atherosclerosis, diminished B1a lymphocytes and B1a-produced natural IgM antibodies are rational explanations for aggravated atherosclerosis in splenectomized mice. Therefore, we hypothesized that B1a lymphocytes, unlike B2 lymphocytes, are an atheroprotective B-lymphocyte subset.

To test this hypothesis, we revisited the splenectomy-aggravated atherosclerosis experiments. We confirmed that splenectomy reduced peritoneal B1a lymphocytes and at the same time aggravated atherosclerosis in ApoE<sup>−/−</sup> mice. Furthermore, we found that adoptive transfer of B1a lymphocytes repopulated B1a lymphocytes in the peritoneum and potently attenuated atherosclerosis aggravated by splenectomy. We have also demonstrated that IgM produced by B1a lymphocytes is required for their atheroprotective action.

**Methods**

**Animals**

All animal procedures and protocols were approved by the Animal Ethics Committee of the Alfred Medical, Research, and Education Precinct, Prahran, Melbourne, Australia. All male mice were bred on a C57BL/6 background. ApoE<sup>−/−</sup> and C57BL/6 mice were from the Precinct Animal Centre, Alfred Medical, Research, and Education Precinct. Ly5.1 mice were from the Walter and Eliza Hall Institute, Melbourne, Australia. Secretory IgM-deficient (sIgM<sup>−/−</sup>) mice generated by Jianzhu Chen, Massachusetts Institute of Technology, were supplied by Troy Randall, University of Rochester.

ApoE<sup>−/−</sup> mice were subjected to either splenectomy or sham operation and fed a high-fat diet with 21% fat and 0.15% cholesterol (Specialty Feeds, Glen Forrest, Western Australia) for 8 weeks. At the end of experiments, mice were killed and peritoneal fluids collected for differential cell analysis by flow cytometry; aortic roots were frozen in OCT embedding medium, and plasma was kept in a −80°C freezer.

**Cell Isolation**

Peritoneal fluid and/or spleens were collected from donor mice. CD19<sup>+</sup> CD5<sup>+</sup> or CD19<sup>−</sup> CD5<sup>+</sup> peritoneal B1a B lymphocytes were isolated by FACSAria (BD Biosciences, San Jose, CA; Figures 1A and 1B), and splenic B2 B lymphocytes were magnetically purified by AutoMACS (Miltenyi Biotec, Auburn, CA; Figure 1C). Cell viability by the Trypan blue exclusion method was >95%.

**Flow Cytometry**

Peritoneal B- and non-B-lymphocyte populations were analyzed with fluorochrome-conjugated antibodies (BD Pharmingen, San Diego, CA) on a BD FACSCanto II (BD Biosciences) as described previously.
Histological Lesion Analysis at Aortic Roots
Atherosclerotic lesion sizes were assessed with oil red O and collagen content with picrossirius red as described previously. To analyze necrotic core areas, aortic root atherosclerotic lesions were stained with hematoxylin and eosin.

Immunohistochemical Analysis at Aortic Roots
Macrophages, smooth muscles, and oxidized low-density lipoprotein (oxLDL) at aortic root atherosclerotic lesions were assessed by immunohistochemical analyses as described previously. Apoptotic cells were identified by terminal dUTP nick end-labeling (TUNEL).

Enzyme-Linked Immunosorbent Assay
Plasma IgG and IgM levels were assessed at 4 and 8 weeks after splenectomy as described previously. A modified ELISA protocol adapted from Caligiuri et al was used to measure anti-malondialdehyde-oxidized (MDA)-LDL. IgM antibody with human LDL modified and assessed by spectrophotometry as described previously.

Lipid Profiles
Plasma lipids (total cholesterol, high-density lipoprotein cholesterol, very-low-density lipoprotein/LDL cholesterol, and triglycerides) were measured by a standard commercial enzymatic assay as described previously.

Statistical Analysis
GraphPad Prism 4 software was used for statistical analyses. Results are presented as mean±SEM. One-way ANOVA with Newman-Keuls post hoc test was used for comparisons of multiple groups of ≥3. A 2-tailed unpaired Student *t* test was used for comparison of 2 groups. A 1-tailed unpaired Student *t* test was used for confirmatory transfer experiments, with B1a lymphocytes selected for CD19 CD5 expression and for Ly5.1 expression. *P*<0.05 was considered significant.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Splenectomy Selectively Decreases Peritoneal B1a Lymphocytes Without Affecting Other Lymphocyte Populations, Body Weight, or Plasma Lipids
Because splenectomy has been reported to reduce peritoneal B1a lymphocytes in the C57BL/6 mouse, we investigated whether splenectomy causes a similar B1a lymphocyte reduction in the ApoE−/− mouse. Splenectomy depleted CD22+ CD5+ B1a lymphocytes in the peritoneal cavity by 68% compared with sham-operated mice (Figures 2A and 2B). B2 and other lymphocytes in the peritoneal cavity (Figures 2B and 2C) were unaffected.

Increased plasma lipids are important initiating factors in the early stage of atherosclerosis. We questioned whether splenectomy has an effect on these lipids in the ApoE−/− mouse. Both body weight (Figure 2D) and lipid profile (Figure 2E) imposed by an 8-week high-fat diet were unaffected by splenectomy, because they were similar to the body weight (mean±SEM 26.81±1.11 g) and lipid profile (mean±SEM: total cholesterol 21.8±1.73 mmol/L, high-density lipoprotein cholesterol 4.12±0.26 mmol/L, very-low-density lipoprotein/LDL cholesterol 17.34±1.42 mmol/L, triglycerides 3.02±0.25 mmol/L) of ApoE−/− mice fed a high-fat diet for 8 weeks.

Splenectomy Increases Atherosclerotic Lesions but Does Not Increase Lesion Smooth Muscle and Collagen Content
In agreement with previous reports, splenectomized mice exhibited a 35% increase in oil red O–stained atherosclerotic lesions (Figure 3A) and a 49% increase in CD68 macrophage accumulation (Figure 3B) compared with sham-operated mice; however, macrophage accumulation expressed as a percentage of total atherosclerotic lesion area was unaffected by splenectomy (Figure 3C). We next examined smooth muscle and collagen content in atherosclerotic lesion to determine whether splenectomy alters plaque stability. Smooth muscle and collagen content stained by α-actin smooth muscle antibody and picrossirius red, respectively, were unaffected (Figures 3C and 3D).

Adoptive Transfer of B1a Lymphocytes Reconstitutes the Peritoneal Compartment
Given that splenectomy reduced peritoneal B1a lymphocytes, we first set out to ascertain whether adoptive transfer of B1a lymphocytes to splenectomized mice would reconstitute the peritoneal compartment. We isolated CD22+ CD5+ B1a lymphocytes (purity >96%; Figure 1A) from the peritoneum of C57BL/6 mice. Because peritoneal B1a lymphocytes require the spleen for survival, and splenectomy reduced their numbers after 1 week and sustained this reduction up to 3 weeks, we transferred 107 peritoneal B1a lymphocytes at weeks 1, 4, and 7 after splenectomy to maintain a pool of replenished peritoneal B1a lymphocytes. We controlled the experiment with transfers of the same numbers of spleen B2 lymphocytes (purity >98%; Figure 1C) and PBS in a similar manner. Transfer of B1a lymphocytes, but not B2 lymphocytes or PBS, reconstituted peritoneal B1a lymphocytes by approximately 67% compared with sham-operated mice and increased this population by approximately 90% compared with splenectomized mice (Figure 2A).

Transfer of B1a but Not B2 Lymphocytes Potently Reduces Atherosclerosis Aggravated by Splenectomy
Next, we examined atherosclerotic lesions at the aortic root to determine whether adoptively transferred B1a lymphocytes can influence the augmented atherosclerosis in splenectomized mice. After assessment of atherosclerotic lesion size by lipid accumulation with oil red O stain, transfer of B1a lymphocytes was shown to potently decrease atherosclerotic lesions by ~70% compared with sham-operated mice and by ~80% compared with splenectomized mice (Figure 3A). Similarly, CD68 macrophage accumulation was also markedly decreased by ~60% compared with sham-operated mice and by ~75% compared with splenectomized mice (Figure 3B), although CD68 macrophage accumulation expressed as a percentage of total atherosclerotic lesion area was unaffected by splenectomy (Figure 3C). There were no differences in body weights and plasma lipids among the experimental groups (Figures 2D and 2E).

The Vast Majority (>98%) of CD22+ CD5+ Peritoneal B1a Lymphocytes Are CD19+ CD5+
We used anti-CD22 and anti-CD5 antibodies to isolate peritoneal B1a lymphocytes. Because CD22 has been reported to be expressed on murine T cells, we compared the phenotype of peritoneal B1a lymphocytes isolated with CD22+ CD5+ antibodies to those isolated with CD19+ CD5+ antibodies (Figure 4A). The vast majority (>98%) of CD22+ CD5+ peritoneal B lymphocytes expressed CD19 antigen but not CD3, whereas 95% of CD19+ CD5+ peritoneal B lymphocytes expressed CD22. Both populations expressed CD11b but not CD23. Furthermore, adoptive transfer to splenectomized mice of peri-
toneal B1a lymphocytes isolated by anti-CD19, anti-CD3, anti-CD5, and anti-CD11b antibodies gave very similar results (Figure 4B) to those observed with transfer of CD22+/H11001CD5+/H11001Bla lymphocytes (Figure 3A). To assess the contribution of donor Bla lymphocytes to reconstitution of this population, we isolated peritoneal CD3+/H11002CD19+/H11001CD5+/H11001CD11b+/H11001Bla lymphocytes from allelic Ly5.1 mice for transfer to splenectomized mice (n=3). Assessment of donor Ly5.1+ cells in the B1a lymphocyte population at the end of an 8-week high-fat diet showed that they constituted 47.0±8.0% (mean±SEM) of this population (Figure 4C). These findings are consistent with the percentage increase in this population after transfer of Bla cells to splenectomized mice compared with their percentage in splenectomized mice (Figure 2A).

IgM in Plasma and Lesions Is Reduced by Splenectomy and Markedly Increased by Transfer of B1a Lymphocytes

Because the majority of circulating natural IgM antibodies are constitutively produced by B1a lymphocytes throughout life,13 and these antibodies react to oxLDL, we examined whether IgM and oxLDL-specific IgM antibody produced by B1a lymphocytes are selectively affected by splenectomy. Plasma levels of IgM assessed at 4 and 8 weeks after splenectomy were decreased by 45% in splenectomized mice compared with sham-operated mice (Figures 5B and 5D). Plasma IgM levels of ApoE mice fed a high-fat diet for 8 weeks and normal C57Bl/6 mice (mean±SEM 0.82±0.14 OD450) were almost identical to those of sham-operated mice.
Figure 3. Splenectomy increases atherosclerosis development in ApoE<sup>−/−</sup> mice. At the end of an 8-week high-fat diet, splenectomized (SX) mice showed (A) increased oil red O–stained atherosclerotic lesions and (B) CD68-stained macrophage areas. However, macrophage (B), lesion smooth muscle (C), and collagen content (D) expressed by lesion size were unaffected by splenectomy. Transfer of WT B1a lymphocytes but not slgM<sup>−/−</sup> B1a lymphocytes, B2 lymphocytes, or saline decreased postsplenectomy aggravated atherosclerotic lesions and macrophage content (A, B). Graphs present mean±SEM values (n=7–8). ***P<0.001, **P<0.01, *P<0.05, 1-way ANOVA with Newman-Keuls post hoc test (A, B) and 2-tailed unpaired t test (C, D). Bars, 100 μm in A, B, and 200 μm in C. SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-slgM<sup>−/−</sup> B1a, splenectomy with slgM-deficient B1a transfer.
In contrast, plasma levels of IgG were unaffected (Figure 5A). Splenectomized mice also showed decreased IgM plasma level against MDA-LDL compared with sham-operated mice (Figure 5C).

Next, we determined whether adoptively transferred B1a lymphocytes can restore IgM and oxLDL-specific IgM levels decreased by splenectomy. Plasma levels of IgM assessed at 4 and 8 weeks after splenectomy increased by 68% in splenectomized mice into which B1a lymphocytes had been transferred, to levels that were almost the same as in sham-operated mice (Figures 5B and 5D). Plasma IgM levels from splenectomized mice into which B2 lymphocytes or PBS had been transferred did not show any difference compared with splenectomized mice (Figure 5B). Transfer of B1a lymphocytes also restored plasma MDA-LDL-specific IgM levels decreased by splenectomy (Figure 5C). Plasma levels of IgG were unaffected in all mice in the transfer experiments (Figure 5A).

Natural IgM antibodies, abundant in the circulation, can also be found in the subintimal space of atherosclerotic lesions. Our demonstration of a direct correlation between plasma IgM level and B1a lymphocytes led us to examine atherosclerotic lesions for IgM deposits. IgM deposits in atherosclerotic lesions were decreased by 58% after splenectomy (Figure 6). After transfer of B1a lymphocytes to splenectomized mice, lesion IgM deposits were increased by 100% compared with splenectomized mice, to a level similar to that in sham-operated mice. In contrast, splenectomized mice into which B2 lymphocytes or PBS had been transferred exhibited similar percentages of areas of lesion IgM deposits as splenectomized mice (Figure 6).
B1a Lymphocytes That Cannot Secrete IgM Fail to Reduce Atherosclerosis Augmented by Splenectomy and Do Not Increase Plasma or Lesion IgM Despite Reconstitution of the Peritoneal Compartment

The observation that splenectomy-aggravated atherosclerosis is accompanied by decreased plasma and lesion IgM whereas transfer of WT but not sIgM-deficient B1a lymphocytes increased plasma IgM and MDA-oxLDL IgM almost to levels in sham-operated mice. Transfer of WT-B2 lymphocyte and PBS did not affect plasma IgM levels or MDA-oxLDL-IgM antibodies. Plasma IgM levels were reduced at 4 weeks after splenectomy and remained at this decreased level at 8 weeks. WT B1a lymphocytes restored plasma IgM levels. Graphs present mean±SEM values (n=7–8 in panels A–C and n=3–4 in panel D). **P<0.01, *P<0.05, 1-way ANOVA with Newman-Keuls post hoc test (A–C) and 1-tailed unpaired t test (D). SX-WTB1a indicates splenectomy with WT B1a transfer; SX-WTB2, splenectomy with WT B2 transfer; SX-PBS, splenectomy with PBS transfer; SX-sIgM/B1a, splenectomy with sIgM-deficient B1a transfer.

To further assess the role of IgM in atherosclerosis, we determined the levels of IgM in the circulation and in atherosclerotic lesions of splenectomized mice into which sIgM−/− B1a lymphocytes had been transferred. As expected, the levels of plasma IgM and oxLDL-specific IgM antibody in these mice were decreased to levels similar to splenectomized mice (Figures 5B and 5C) without affecting plasma IgG (Figure 5A). Furthermore, the transferred sIgM−/− B1a lymphocytes also failed to increase IgM deposits in lesions, which contrasted with the effects of transferred wild-type (WT) B1a lymphocytes (Figure 6).

Necrotic Cores in Atherosclerotic Lesion Are Increased by Splenectomy and Reduced by Transfer of B1a Lymphocytes

We next examined atherosclerotic lesions for necrotic cores, because it has been suggested that a major function of natural
IgM is to clear apoptotic cells\textsuperscript{21} by binding to oxidation-specific epitopes.\textsuperscript{19} We assessed the size of the necrotic core identified as acellular areas after hematoxylin-and-eosin staining of atherosclerotic lesions.\textsuperscript{15} Necrotic core areas in atherosclerotic lesions increased by 19% after splenectomy and decreased by 14% with the transfer of B1a lymphocytes. In contrast, sIgM\textsuperscript{−/−} B1a lymphocytes failed to decrease the necrotic core area augmented by splenectomy (Figure 7A).

Given that approximately 30% of natural IgM antibodies bind to oxidation-specific epitopes,\textsuperscript{22} we next examined atherosclerotic lesions for oxLDL using an antibody to MDA-LDL. OxLDL in lesions was increased by splenectomy and reduced by transfer of B1a lymphocytes (Figure 7B).
tomy and decreased by transfer of WT B1a lymphocytes (Figure 7B).

Next, we assessed TUNEL-positive apoptotic cells in atherosclerotic lesions, because natural IgM antibodies are responsible for apoptotic cell clearance in chronic inflammation.14 Apoptotic cells were increased in splenectomized mice and decreased by transfer of WT B1a lymphocytes (Figure 8).

**Discussion**

B1 lymphocytes differ from B2 lymphocytes in origin, distribution, surface markers, and function. Whereas conventional B2 lymphocytes have important roles in adaptive immunity, B1 lymphocytes have key roles in innate immunity. B1 lymphocytes are self-replenishing, long-lived B lymphocytes that reside mainly in serosal cavities, such as in the peritoneum and the pleura. They are a minor B-lymphocyte population in the circulation and in the spleen23,24 and comprise B1a (CD11b+, CD5+) and B1b (CD11b+, CD5+) subsets. B1a lymphocytes are implicated in first-line defense against bacterial and viral infections.25 They respond rapidly and strongly to pathogen-derived products such as phosphorylcholine but poorly to receptor-mediated activation.13,25,26 B1a lymphocytes rarely enter germinal centers to undergo affinity maturation and are thus highly restricted in their ability to produce high-affinity antibodies. Instead, in humans and mice, they produce low-affinity, polyreactive IgM antibodies throughout life. These are called “natural antibodies” because their levels in germ-free mice are virtually identical to those of mice housed in conventional facilities.27 Thus, natural antibodies are generated in the absence of external antigenic stimulation and are selected presumably by endogenous ligands.28 These antibodies are produced mainly, if not exclusively, by B1a lymphocytes and constitute most, if not all, circulating IgM.13

Our observation that splenectomy depleted peritoneal B1a lymphocytes and augmented atherosclerosis, assessed by oil red O–stained lipid accumulation, without affecting body weight and hypercholesterolemia provides a direct relationship between peritoneal B1a lymphocytes and atherosclerosis. Macrophage accumulation assessed by CD68-stained areas was also increased by splenectomy and decreased by WT but not by sIgM-deficient peritoneal B1a lymphocytes. However, the percentage of CD68+ macrophage content expressed in relation to total lesion area remained unchanged, which suggests little if any effect on lesion composition. The observation that splenectomy did not affect collagen or smooth muscle content of atherosclerotic lesions further supports the notion that splenectomy-induced depletion of B1a lymphocytes did not affect lesion composition or plaque stability. Atherosclerosis aggravated by splenectomy-induced B1a lymphocyte depletion coupled with reduction of the aggravated atherosclerosis by repletion of the B1a lymphocyte population by adoptive transfer provides compelling evidence for an atheroprotective role for B1a lymphocytes. Reconstitution of peritoneal B1a lymphocytes should have reduced atherosclerosis to the level in sham-operated mice. Instead, the transferred B1a lymphocytes reduced atherosclerotic lesions to a level far below that in sham-operated mice. These results highlight the potency of the B1a lymphocytes in suppressing atherosclerosis development.

The present results implicate natural IgM antibodies produced by B1a lymphocytes and deposited in atherosclerotic lesions in mediating protection against lesion development. Thus, we showed that plasma IgM and lesion IgM deposits were decreased on depletion of peritoneal B1a lymphocytes after splenectomy, without affecting plasma IgG. Only the adoptive transfer of B1a lymphocytes, and not B2 lymphocytes, reversed plasma and lesion IgM, with 67% reconstitution in the peritoneal cavity compared with sham-operated mice. The observation that transfer of B1a lymphocytes that cannot secrete IgM failed to increase plasma and lesion IgM and to attenuate atherosclerosis despite reconstitution of the peritoneal compartment confirms the key role played by natural IgM produced by B1a lymphocytes in protection against atherosclerosis development. Our
findings are consistent with B1a lymphocytes being responsible for generating the majority of natural IgM and with the report that genetically manipulated asplenic mice and splenectomized mice did not produce plasma IgM. Transfer of fetal liver cells, not bone marrow cells, from both Hox11–/– or C57BL/6 donors regenerated peritoneal B1a lymphocytes. These regenerated B1a lymphocytes produced IgM antibodies against streptococcal polysaccharides regardless of the immunization route, in contrast to splenectomized, asplenic mice and irradiated Rag–2–/– mice transferred with bone marrow that failed to produce these antibodies.

Circulating IgM antibodies comprise B1a-produced natural IgM and B2-produced antigen-specific IgM. The former is generated in the absence of antigen stimulation, whereas the latter requires antigen stimulation. Both natural and antigen-specific IgM antibodies are required for optimal protection against microbial antigens. Natural IgMs play an important role in initial defense, whereas antigen-specific IgMs act together with natural IgGs to enhance IgG responses in late immune responses. Our finding that splenectomy decreased both natural IgM antibodies and IgM antibodies specific to oxLDL is consistent with selective depletion of B1a lymphocytes by splenectomy. The observation that transfer of WT B1a but not slgM-deficient B1a lymphocytes rescued postsplenectomy-aggravated atherosclerosis indicates that natural IgM produced by B1a lymphocytes protects against atherosclerosis development.

The present data suggest possible mechanisms by which natural IgMs produced by B1a lymphocytes and deposited in atherosclerotic lesions mediate atheroprotection. The finding that atherosclerotic lesions aggravated by splenectomy-induced B1a lymphocyte depletion are accompanied by reduced IgM deposits in lesions and increased necrotic core, oxLDL, and apoptotic cells in lesions suggests that IgM deposited in lesions plays a role in clearing apoptotic cells and proinflammatory oxLDL. This suggestion is further supported by the finding that transfer of WT B1a lymphocytes to splenectomized mice is accompanied by increased IgM deposits in lesions and decreased necrotic core, oxLDL, and apoptotic cells. Failure of transferred slgM–/– B1a lymphocytes to increase lesion IgM and reduce necrotic cores is consistent with this suggestion. Together, the present data support a scavenger role for natural IgM in clearing apoptotic cell debris and oxLDL. Natural IgM has been suggested to bind to apoptotic cells through oxidation-specific epitopes, resulting in clearance of apoptotic cells and cellular debris from atherosclerotic lesions. Approximately 30% of these IgM bind to model oxidation-specific epitopes and to atherosclerotic lesions. Natural IgM also blocks oxLDL uptake by macrophages, preventing foam cell formation.

IgM antibodies assessed at 4 and 8 weeks after splenectomy showed that the WT B1a-transferred group had approximately the same IgM level as the sham-operated group. The significant and persistently lower plasma IgM level as a result of splenectomy-induced depletion of natural IgM-antibody-producing B1a lymphocytes is consistent with the short half-life of IgM (2 days). Transfer of WT B1a lymphocytes reconstituted the peritoneal compartment and restored plasma IgM level to almost the same level as in sham-operated mice. In contrast, transfer of IgM-deficient B1a lymphocytes failed to increase plasma IgM level despite reconstitution of peritoneal B1a lymphocytes. Enhanced reconstitution by peritoneal slgM–/– B1a lymphocytes may reflect homeostatic expansion in an slgM-deficient environment.

Identification of IgM-secreting B1a lymphocytes as an atheroprotective B-lymphocyte subset coupled with our previous identification of B2 lymphocytes as an atherogenic population highlights the opposing roles played by these B-lymphocyte subsets in atherosclerosis. We previously reported that transfer of B2 lymphocytes to lymphocyte-deficient mice aggravated atherosclerosis; however, transfer of B2 lymphocytes did not aggravate atherosclerosis in splenectomized mice. This outcome is probably because the transferred naïve B2 lymphocytes (3×10⁶ cells) comprise only a small fraction of the host’s total reservoir of B2 lymphocytes.

Our findings have therapeutic implications. They indicate that a rational approach in B lymphocyte-depletion therapy for inflammatory diseases including atherosclerosis should be directed toward selective depletion of damaging B lymphocytes while sparing protective B lymphocytes such as B1a lymphocytes. The findings also suggest that therapeutic strategies directed toward expansion of atheroprotective B1a lymphocytes can be exploited to attenuate atherosclerosis development and progression. Our findings lend credence to the search for a protective vaccine in atherosclerosis.

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Disclosures
None.

References
Novelty and Significance

**What Is Known?**

- B lymphocytes have been reported to reduce atherosclerosis, but the specific subset that exerts this effect has not been identified previously.
- Depletion of B lymphocytes by anti-CD20 antibody decreased atherosclerosis, whereas adoptive transfer studies identified B2 lymphocytes as a damaging B-lymphocyte subset.
- Splenectomy aggravated atherosclerosis and depleted B1a lymphocytes, whereas IgM was atheroprotective.

**What New Information Does This Article Contribute?**

- Peritoneal B1a lymphocytes are an atheroprotective B-cell subset.
- B1a lymphocytes protect against atherosclerosis development by producing natural IgM that circulates in the blood and is deposited in atherosclerotic lesions.
- IgM deposits in atherosclerotic lesions remove dead cells from lesions.

B lymphocytes have been reported to be atheroprotective. However, B lymphocytes are heterogeneous, and although the B2 subset has been identified as atherogenic, the identity of the atheroprotective B-lymphocyte subset remains unknown. Here, we have identified IgM-producing B1a lymphocytes as an atheroprotective B-lymphocyte subset based on the following salient observations: (1) B1a depletion by splenectomy aggravated atherosclerosis, accompanied by reduced plasma and lesion IgM and increased dead cells in lesions; (2) B1a repletion by their transfer to splenectomized mice reduced atherosclerosis, increased plasma and lesion IgM, and reduced dead cells in lesions; (3) transfer of B1a lymphocytes, which cannot produce IgM, failed to protect against atherosclerosis. Our data provide the novel concept that B1a lymphocytes are atheroprotective by producing natural IgM antibodies deposited in atherosclerotic lesions that remove dead cells from lesions. This process explains the exaggerated atherosclerosis after splenectomy and an atheroprotective role of IgM. The significance of this new knowledge lies not only in providing a better insight into the role of B-lymphocyte subsets in atherosclerosis, but also in its potential clinical translation. Our findings suggest that therapeutic strategies directed toward amelioration of atherosclerosis should be directed toward deletion of damaging B2 lymphocytes and/or expansion of protective B1a lymphocytes.
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Supplemental Materials

Splenectomy

In splenectomy experiments, spleens of 6-8 week-old ApoE<sup>−/−</sup> mice were removed surgically under aseptic conditions. Briefly, under anesthesia with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg), a 10-mm left flank incision was made to expose the spleen and the whole spleen was removed using diathermy. The peritoneum and skin were closed separately using 2-0 monofilament suture after checking for any hemorrhage in the abdominal cavity. Upon subcutaneous injection of Atipamezole HCl (antisedan 100mg/kg), mice were placed in 37˚C recovery chambers before they were returned to their cages. Sham-operation was performed according to the splenectomy procedure but without removing the spleen. For transfer experiments, purified 10⁵ B1a lymphocytes were intravenously transferred via the tail vein.

Cell isolation

Peritoneal fluid and/or spleens were collected from donor mice<sup>1</sup>. After lysing red blood cells and preparing single cell suspensions, peritoneal B1a lymphocytes were isolated by FACS Aria (BD Biosciences) using anti-CD22 and anti-CD5 antibodies (BD Pharmingen) (Fig. 1a). In some experiments, anti-CD3, anti-CD19, anti-CD5 and anti-CD11b antibodies (BD Pharmigen) were used to isolate peritoneal B1a lymphocytes (Fig. 1b). Spleen B2 lymphocytes were sorted magnetically by negative selection using a B lymphocyte isolation kit (Miltenyi Biotec) (Fig. 1c). Purity of cells was checked by anti-CD22/anti-CD19 and anti-CD5 antibodies (BD Pharmingen) on FACS Aria (BD Biosciences) or FACS Cantos II (BD Biosciences). Cell viability checked by trypan blue exclusion method was >95%. To assess the contribution of donor B1a lymphocytes to the reconstitution of this population, peritoneal CD3<sup>−</sup>CD19<sup>+</sup>CD5<sup>−</sup>CD11b<sup>+</sup> B1a lymphocytes were also isolated from allelic Ly5.1 mice, and transferred to splenectomised mice. B1a lymphocytes isolated from these mice at the end of the 8 week experiment were then assessed for Ly5.1<sup>+</sup> donor cells in this population.

Flow cytometry

B and non-B lymphocyte populations in peritoneal fluid were analyzed using fluorochrome-conjugated antibodies (BD Pharmingen) on BD FACSCanto II (BD Biosciences) as described<sup>1</sup>.

Histological lesion analysis at aortic roots

The heart and proximal aorta were dissected from mice, embedded in OCT compound (Tissue-teck) and frozen at -80°C. Frozen sections (6μm) were cut from the aortic sinus, defined as the region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off<sup>2</sup>. The aortic sinus was evaluated because this region of the aorta is particularly susceptible to development of atherosclerosis in mice fed a HFD<sup>2</sup>. Atherosclerotic lesion sizes were assessed using oil red-O and collagen content using picrosirius red as previously described<sup>1</sup>. To analyse necrotic core areas, aortic root atherosclerotic lesions were stained with hematoxylin and eosin (H&E) stain. Acellular (non-stained) areas were measured by Optimas software and expressed as a percentage of lesion areas<sup>3</sup>.

Immunohistochemical analysis

Immunohistochemical analyses were performed at aortic root atherosclerotic lesions as described<sup>1</sup>. Rat anti-mouse CD68 antibody (Serotec, Raleigh, NC), rabbit anti-α-actin smooth muscle antibody (Abcam, Cambridge, UK) and mouse anti-MDA- oxidized low-density lipoproteins (oxLDL) antibody (Abcam, Cambridge, UK) were used to detect macrophages, smooth muscles, and oxLDL in atherosclerotic lesions.
Some sections were used to identify apoptotic cells using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) system and alkaline phosphatase [in-situ cell death detection kit-AP assay (Roche)] according to the manufacturer’s instruction.

Detection of plasma IgG and IgM

Plasma levels of IgG and IgM were measured by enzyme linked immunosorbent assay (ELISA) as described¹. In time-course analysis, IgM levels were assessed at 4 and 8 weeks after splenectomy.

Detection factors used for sample preparation were $10^5$ for plasma IgG and $10^4$ for IgM. To determine plasma immunoglobulins titers using ELISA, 50 μl of anti-mouse immunoglobulin (1μg/ml) was used to coat 96-well ELISA plates overnight at 4°C. After blocking with 1% bovine serum albumin, duplicate samples of 50 μl of plasma (diluted 1:105 for total Ig and IgG and 1:104 for IgM) was added into ELISA plates for 2 hours at room temperature. Respective secondary anti-mouse antibodies conjugated with horseradish peroxidase were added into the wells, followed by addition of TMB substrate for colour development. The OD at 450 nm was read by ELISA reader.

Measurement of anti–malondialdehyde(MDA)-LDL IgM antibody

Modification of human LDL (Calbiochem, Darmstadt, Germany) was carried out and assessed by spectrophotometer as described². A modified ELISA protocol adapted from Caliguiri et al.³ was used to measure anti-MDA-LDL IgM antibody. Briefly, MDA-LDL and native LDL were used to coat 96-well ELISA plates at 50μl of 10 μg/ml overnight at 4°C. Duplicate samples of 50 μl mouse plasma diluted 1:80, 1:320 and 1:1280 were added into the ELISA plates for 1 hour at 37°C after blocking with 1% BSA, followed by addition of 1:10,000 diluted anti-mouse IgM (BD Pharmingen) antibody conjugated with HRP. Color was developed by addition of TMB solution. Plates were read at 450 nm wavelength. MDA-LDL–specific IgM antibody was determined by subtracting the native LDL OD from the oxLDL OD.

Lipid profiles

Plasma lipids (total cholesterol, HDL-cholesterol, VLDL/LDL-cholesterol and triglycerides) were measured by a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyser, with reagents and calibrators supplied by Beckman Coulter Diagnostics Australia as described¹.

Statistical analysis

GraphPad Prism 4 software was used for statistical analyses. Results are presented as mean ± SEM. One-way ANOVA with Newman-Keul post-hoc test was used for comparisons of multiple groups of ≥3. Two-tailed unpaired student t-test was used for comparison of 2 groups. One-tailed unpaired student t-test was used for confirmatory transfer experiments with Bla lymphocytes selected for CD19⁺CD5⁻ expression and for congenic Ly5.1 expression. P values were considered significant at P<0.05.

