Macrophage-specific expression of class A scavenger receptors in LDL receptor$^{-/-}$ mice decreases atherosclerosis and changes spleen morphology

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Abstract **Class A scavenger receptors (SR-A) have been implicated in the atherogenic process, although there have been conflicting reports as to their specific effect on the development of lesions. In part, this discord may arise because of the variable contribution of SR-A in the several cell types known to express this protein. To determine the effects of macrophage-specific SR-A expression in the atherogenic process, transgenic mice were created using the chicken lysozyme (lyso) promoter to drive expression of bovine SR-A (bSR-A). To express this gene in an atherosclerosis-susceptible strain, bone marrow cells from transgenic and non-transgenic littermates were used to repopulate lethally-irradiated female LDL receptor (LDLr)$^{-/-}$ mice. Following hematopoietic engraftment, mice were placed on a diet enriched in saturated fat and cholesterol. After 8 weeks, there was a modest, but statistically significant reduction in serum total cholesterol in LDLr$^{-/-}$ mice repopulated with lyso-bSR-A transgenic cells, due to decreased LDL-cholesterol. The extent of atherosclerosis was reduced in both cross-sectional analysis of the aortic root and en face analysis of the intimal surface of the aortic arch. In addition to changes in atherosclerosis, lyso-bSR-A repopulated LDLr$^{-/-}$ mice had a marked increase (3.6×) in spleen weights and a disruption of spleen white pulp formation. Therefore, macrophage-specific overexpression of SR-A resulted in reduced atherosclerosis in two vascular beds, reduced serum cholesterol concentrations, and changed the morphology of the spleen.**—Whitman, S. C., D. L. Rateri, S. J. Szilvassy, J. A. Cornicelli, A. Daugherty. **Macrophage-specific expression of class a scavenger receptors in LDL receptor$^{-/-}$ mice decreases atherosclerosis and changes spleen morphology. *J. Lipid Res.* 2002.** 43: **1201–1208.**

Supplementary key words bone marrow transplantation • low density lipoprotein • immune function

Class A scavenger receptors (SR-A) recognize a wide variety of polyanionic ligands including modified lipoproteins, phosphatidylserine, lipopolysaccharide, and lipoteichoic acid (1). SR-A has additional roles including adhesion to substrata (2–4) and lymphocytes, (5) and activation of intracellular signaling (6, 7). This wide array of biological properties has led to a hypothesized role for SR-A in several disease states, particularly atherosclerosis and host defense. The most striking evidence that demonstrates a role for this receptor in these diseases comes from SR-A deficient mice that were crossbred onto an apolipoprotein E (apoE)$^{-/-}$ background. Deficiency of SR-A resulted in reduced atherosclerosis in the aortic root, but increased susceptibility to infection by *Listeria monocytogenes* or herpes simplex virus type-1 (8). Since this initial publication, there has been conflicting data on the effects of SR-A on atherogenesis (8–13).

Originally detected on macrophages (14), SR-A is also present on other cell types, including smooth muscle cells and endothelial cells (15–18). SR-A is regulated by a wide variety of molecules, although receptor regulation is complex and may depend on cell type (1). For example, tumor necrosis factor-α downregulates SR-A in macrophages (19, 20), but upregulates this receptor in smooth muscle cells (21). Therefore, complete SR-A ablation in vivo would result in a complex array of effects on different cell types.

Cell-specific expression of SR-A in transgenic animals is an approach to define the role of this receptor in the components of atherosclerotic lesions. Of the cells present in

Abbreviations: BMT, bone marrow transplant; lyso, lysozyme; SR-A, class A scavenger receptors.

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lesions, a major role of SR-A in atherosclerosis is attributed to its activity in macrophages. There are a limited number of promoters that have been used to drive macrophage-specific transgenic expression. The SR-A promoter itself has been used to overexpress human growth hormone (22) and hormone sensitive lipase (23). A second candidate is the macrosialin promoter that has been used successfully in cultured cells (24), although its use in transgenic animals has not been described. A third strategy uses the chicken lysozyme promoter that has been shown to drive macrophage-specific gene expression in both transgenic mice (25) and rabbits (26, 27).

Recently, we have described the development of transgenic mice overexpressing the type II bovine SR-A (bSR-A) under the control of the chicken lysozyme (lyso) promoter. These mice displayed functional increases in macrophage SR-A activity as defined by enhanced cholesterol esterification in response to acetylated LDL (28). For the present study, we created chimeric mice by irradiation of LDL receptor $(LDLr)^{-/-}$ mice and repopulation with bone marrow stem cells from either lyso-bSR-A transgenic mice or age- and gender-matched non-transgenic littermates. The presence of the LDLr in the donor bone marrow cells does not alter the extent of atherosclerosis in recipient $LDLr^{-/-}$ mice (29–31). The generation of chimeric mice by bone marrow transplantation expedited the creation of SR-A overexpressing mice in an atherosclerosis-susceptible background. Animals engrafted with lyso-bSR-A bone marrow cells had a modest reduction in serum cholesterol and a striking reduction in the extent of atherosclerosis in two vascular beds. In addition, the expression of bSR-A promoted marked hypertrophy of splenic tissue.

MATERIALS AND METHODS

Mice

Lyso-bSR-A transgenic mice were generated directly in a C57BL/6 background as described previously (28). The transgenic mice were bred to C57BL/6 wild-type mice, and littermates were used for comparing transgenic versus non-transgenic mice. As described previously, lyso-bSRA transgenic mice appear normal (28). $LDLr^{-/-}$ mice that were backcrossed 10 times into a C57BL/6 background were purchased from the Jackson Laboratory. All studies were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Bone marrow transplantation

Eight-week-old, female $LDLr^{-/-}$ mice (n = 20) in a C57BL/6 background were maintained on antibiotic-containing water for 1 week before irradiation. These mice were irradiated with a total of 900 Rads from a cesium source (delivered in two equal doses of 450 Rads 3–4 h apart), and donor bone marrow cells (1×10^7) were then injected into a tail vein (32). Mice were maintained on antibiotic-containing water for 4 weeks after transplantation. Six weeks after transplantation, the mice were fed a diet enriched in saturated fat (27% of wt/wt) and cholesterol (0.15%; Teklad) and maintained for an additional 8 weeks.

Lipid and lipoproteins

Serum total cholesterol concentrations were determined with enzymatic assay kits (Wako Chemical Co). Lipoprotein cholesterol distributions in individual serum samples (50 μl) from four mice in each group were resolved by size exclusion chromatography on a Superose 6 column as described previously (33).

RT-PCR and PCR analysis of bone marrow

Total RNA and DNA was isolated from bone marrow using commercially available kits (Promega and Qiagen, respectively). Bovine SR-A mRNA was detected as described previously (28) and β-actin mRNA was detected with commercially available primers (Ambion). Bovine SR-A and LDLr DNA was detected by PCR as described previously (28, 34).

Acquisition of tissues

At the end of the dietary period, mice were anesthetized by methoxyflurane inhalation. Terminal blood samples were collected by puncture of the right ventricle and mice were perfused with phosphate buffered saline (PBS). Hearts were separated from the aorta at the base, embedded in OCT, and frozen at −20°C. Aortic tissue was removed from the ascending aorta to the ileal bifurcation and placed in freshly prepared 4% paraformaldehyde in PBS overnight at room temperature. Spleens were removed, weighed, and segments were frozen in OCT and stored at −20°C.

Quantification of atherosclerotic lesions on the intimal surface of the aorta

Aortic tissues were prepared as described previously (35–37). To quantify the extent of intimal surface covered by grossly discernible lesions, aortic images were captured on a Spot camera (Diagnostic Instruments), and analysis was performed with Image-Pro software (Media Cybernetics). The extent of atherosclerotic lesions was quantified in the arch, thoracic, and abdominal regions of the aorta as described previously.

Quantification of atherosclerotic lesions in the aortic root

Atherosclerotic lesion size in the ascending aorta was determined from four Oil Red O stained serial sections, cut 8 μm thick, and collected at 80 μm intervals, starting at the region where the aortic sinus becomes the ascending aorta as described previously (32, 38, 39). Lesion area, defined as intimal tissue within the internal elastic lamina, was determined using Image-Pro software on images that were created using a Spot camera. The mean lesion area derived from the four serial sections was taken as the average lesion size for each animal.

Immunocytochemistry

Immunocytochemistry was performed as described previously (33, 37) on serial sections of the ascending aorta adjacent to those stained with Oil Red O. The following reagents were used for immunostaining: an antimouse Thy-1.2 monoclonal antibody (PharMingen; catalog # 01011D; 7 μg/ml), a mouse macrophage polyclonal antiserum (Accurate Chemical Co; catalog # AI-AD31240; 1:3000 dilution), an antimouse MARCO monoclonal antibody (Serotec; catalog # MCA1849; 1:100), and an antimouse MHC II monoclonal antibody (Biosource International; catalog # AMU0191; 1:5). Species-specific biotinylated secondary antibodies and a biotin-avidin-peroxidase complex were subsequently incubated with tissues (Vectastain Elite ABC kit, Vector Laboratories). Immunoreactivity was visualized using the red chromagen, 3-amino-9-ethyl carbazole (Biomeda Corp).

Statistics

Data analyses were performed using SigmaStat 2.03 software (SPSS Inc.). Statistical analysis between groups was assessed by Student's *t*-test after testing that the data complied with the constraints of parametric analysis. Values with $P \leq 0.05$ were considered statistically significant.

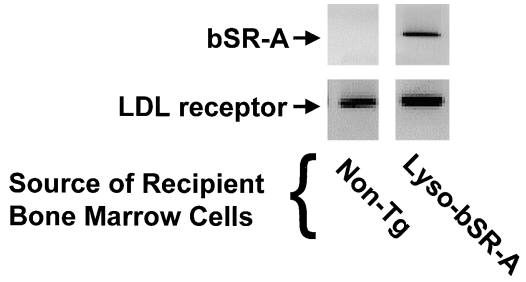

Fig. 1. Verification of engraftment and presence of the transgene in LDL receptor (LDLr)$^{-/-}$ mice that were repopulated with bone marrow cells from non-transgenic or lysosome bovine class A scavenger receptor A (lyso-bSR-A) transgenic donors. DNA was extracted from bone marrow at the end of the experiment and PCR used to detect bSR-A and LDLr DNA. Representative examples are shown.

RESULTS

All mice survived the bone marrow transplant procedure and appeared healthy throughout the study. There were no overt differences in the appearance or general health of non-transgenic or lyso bSR-A repopulated LDLr$^{-/-}$ mice. Body weights, numbers of circulating white cells, red cells, or platelets were not significantly different between the groups (data not shown). Engraftment was verified by PCR detection of the LDLr gene in the bone marrow of all irradiated mice, as was the presence or absence of the bSR-A transgene (**Fig. 1**). Although bSR-A DNA was detected in bone marrow of the mice repopulated with transgenic cells, we were unable to detect mRNA even by RT-PCR (data not shown). Therefore, the lysozyme promoter did not lead to bSR-A expression in cells that are resident in the bone marrow.

Serum cholesterol concentrations were significantly decreased ($P < 0.01$) in the mice repopulated with bone marrow cells from lyso-bSR-A transgenic mice (**Fig. 2A**). To determine the fraction responsible for this decrease, the lipoprotein distribution of cholesterol was analyzed by size exclusion chromatography for each individual mouse. This analysis demonstrated that the decrease in serum cholesterol concentrations was predominantly due to a decrease in the LDL fraction (Fig. 2B).

The extent of atherosclerosis was quantified both by en face analysis of the aortic arch and sequential cross-sectioning of the aortic root. Both measurements demonstrated significantly less atherosclerosis in the aortic root and arch from lyso-bSR-A repopulated mice (**Fig. 3**).

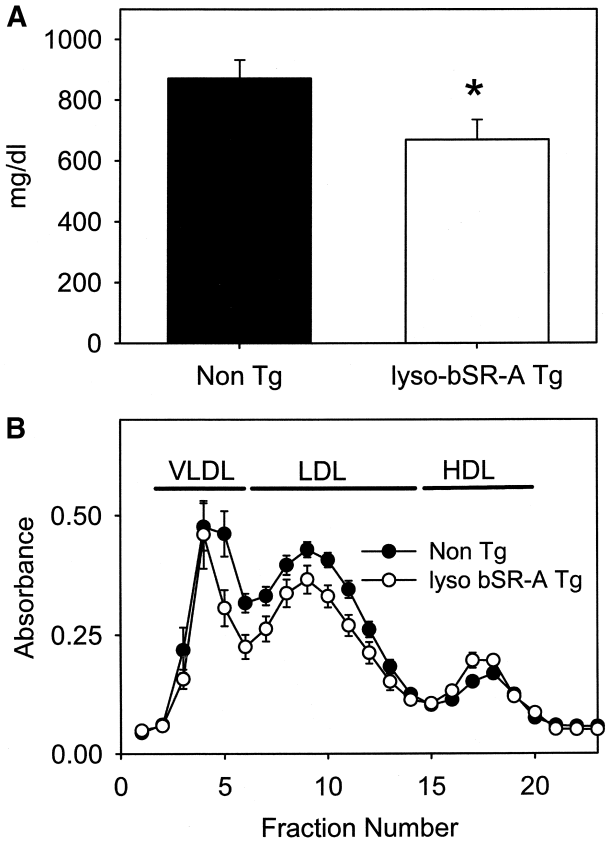

Fig. 2. Characterization of serum cholesterol. A: Total cholesterol concentrations in serum from female LDLr$^{-/-}$ mice receiving bone marrow transplants from either non-transgenic (closed bars; n = 10) or lyso-bSR-A transgenic littermates (open bars; n = 10). Histobars represent means and bars represent SEM. *$P < 0.01$. B: Serum (50 μl) from LDLr$^{-/-}$ mice receiving bone marrow transplants from either non-transgenic (closed circles) or lyso-bSR-A transgenic littermates (open circles) was resolved by size exclusion chromatography using a Superose 6 column. Total cholesterol concentrations were determined in each fraction. Symbols represent the means and bars the SEM of values obtained from serum of four mice.

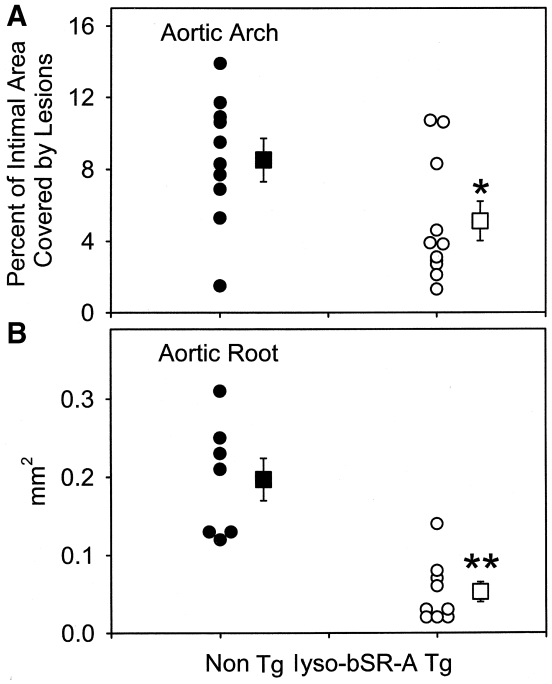

Fig. 3. Quantification of atherosclerosis in LDLr$^{-/-}$ mice receiving marrow from either non-transgenic (closed symbols) or lyso bSR-A transgenic (open symbols) donors. A: The extent of intimal surface covered by grossly discernible lesions was determined from en face preparations of the aortic intima in the arch region. B: Atherosclerotic lesion size was determined from cross-sections of the ascending aorta. Values of individual mice are represented as circles, squares are means, and bars are SEM. *$P < 0.05$, **$P < 0.001$.

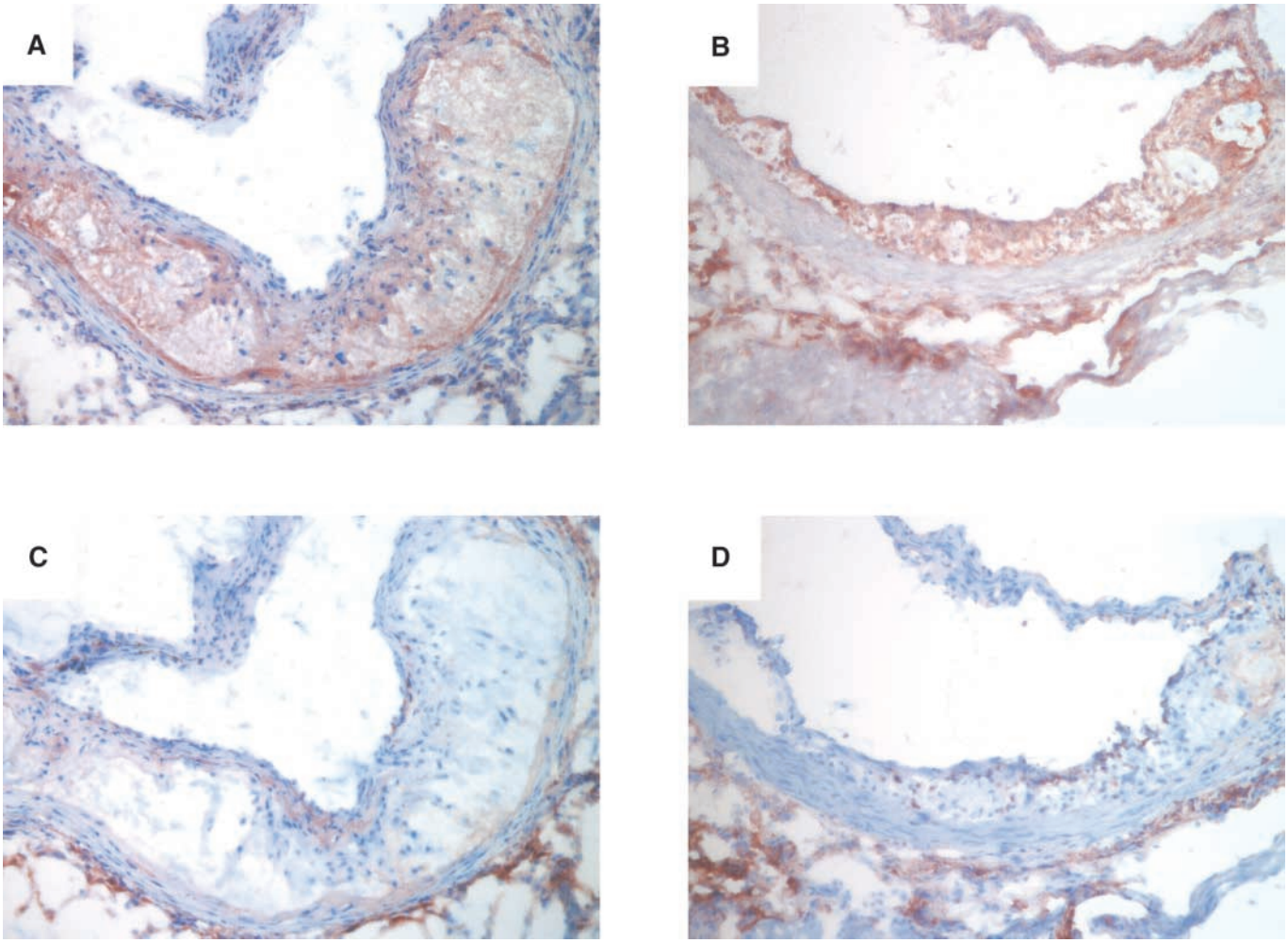

Fig. 4. Representative histological sections from a region where the aortic sinus becomes the ascending aorta of female LDLr$^{-/-}$ mice that were repopulated with bone marrow cells from either non-transgenic (A and C) or lyso bSR-A transgenic mice (B and D). A segment of heart tissue spanning the aortic sinus and ascending aorta was embedded in OCT, sectioned, and immunostained with: A and B: a rabbit antisera to mouse macrophages; C and D: a monoclonal antimouse Thy-1.2 antibody. Magnification 200×.

Given the brief interval of feeding the modified diet, there were insufficient atherosclerotic lesions present in the thoracic or abdominal regions to permit quantification. The reduction was particularly striking in the case of the aortic root, where the extent of atherosclerotic lesions was decreased by 74% (Fig. 3B).

Immunocytochemical analysis of lesions in the aortic root was performed to determine any compositional change in the lesions. Sections from mice repopulated with non-transgenic bone marrow exhibited lesions with fibrous caps that contained cores with sparse nuclei and immunostained uniformly for macrophages (**Fig. 4A**). The atherosclerotic lesions from lyso-bSR-A repopulated mice were considerably smaller as represented quantitatively in Fig. 3. Lesions in the lyso-bSR-A repopulated mice usually lacked prominent fibrous caps and also contained macrophages (Fig. 4B). As described previously, the presence of T lymphocytes in the lesions was mostly restricted to the luminal regions (40), but there was no obvious difference between the groups in the number of these cells present (Fig. 4C, D). MHC class II immunostaining was also similar in lesions of both groups of mice (data not shown).

We also performed a gross tissue survey in both groups of LDLr$^{-/-}$ mice and observed a large increase in the size of the spleens of lyso-bSR-A repopulated mice compared with those repopulated with bone marrow from non-transgenic littermates (468 ± 45 g vs. 128 ± 6 g, $P < 0.001$, respectively; **Fig. 5**). Spleen size was not altered in C57BL/6 lyso-bSR-A mice [97 ± 12 g vs. 96 ± 6 g, (n = 8)] for transgenic and non-transgenic littermates, respectively]. As illustrated in the cross-sections shown in **Fig. 6**, the spleens of the lyso-bSR-A repopulated mice also had an altered morphology. We used an antibody to MARCO, a class A scavenger receptor, that is highly expressed in marginal zone macrophages (41). MARCO immunostaining indicated an expansion of the white pulp (Fig. 6A, B). In white pulp of non-transgenic mice, there was the expected cluster of T lymphocytes (Fig. 6C, E), whereas the Thy-1.2 antigen was not expressed in a specific spatial manner in spleens from the transgenic mice (Fig. 6D, F). While macrophage immunostaining was most prominent in the mar-

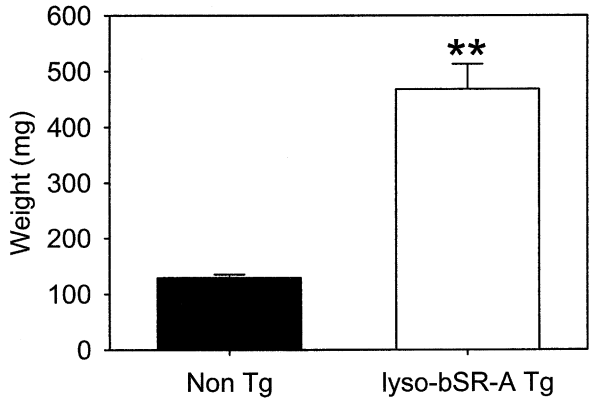

Fig. 5. Weight of spleens from LDLr$^{-/-}$ mice transplanted with bone marrow from non-transgenic (closed histobars) or transgenic littermates (open histobars). Histobars represent the mean of 10 individual mice and bars are SEM. $^{**}P < 0.001$.

ginal zone in spleens from mice repopulated with marrow from non-transgenic littermates, mice expressing bSR-A had a more diffuse distribution of macrophages throughout the spleen (Fig. 6G, H). There were no other grossly discernable differences in other tissues between the two groups of mice.

DISCUSSION

This study demonstrated that macrophage-specific functional overexpression of SR-A in LDLr$^{-/-}$ mice decreased serum concentrations of cholesterol and decreased the severity of atherosclerosis in both the aortic arch and ascending aorta. This decrease in atherosclerosis was associated with changes in lesion composition. In addition, there was a pronounced and unexpected increase in spleen size in LDLr$^{-/-}$ mice that were repopulated with cells from lyso-bSR-A transgenic mice.

We have previously demonstrated that transgenic mice expressing bSR-A under the control of the chicken lysozyme promoter modestly augmented AcLDL stimulated cholesterol esterification in cultured peritoneal macrophages (28). Also, macrophages from these transgenic mice exhibited increased spreading in culture. To determine the extent of transgene protein expression in the atherosclerotic lesions of the chimeric mice generated for this study, we attempted to generate antibodies that distinguished between the native and transgene protein. We developed an bSR-A IgY antibody using a peptide sequence in the α helical coiled coil domain based on reactivity of the monoclonal antibody 2F8 to this domain (42). However, this strategy generated a protein that reacted with both the murine and bovine SR-A proteins. Despite our inability to detect the transgenic protein, our previous characterization of lyso-bSR-A transgenic mice demonstrated that there is a functional increase in SR-A activity (28).

Macrophage specific expression of bSR-A lead to a modest, but statistically significant reduction in serum cholesterol concentrations. A similar finding was noted in mice in which bone marrow cells from mice overexpressing the human SR-A were used to repopulate lethally-irradiated LDLr$^{-/-}$ mice (13). Size exclusion chromatographic analysis of serum demonstrated that this decrease was primarily due to a reduction in LDL cholesterol (LDL-C), with a lesser reduction in VLDL-C. A potential explanation for this decrease could be a removal of modified LDL from plasma. Although modified lipoproteins have been detected in the plasma (43, 44), these studies have demonstrated that these particles are present only in very low concentrations in plasma. Therefore, their removal would probably not significantly influence overall plasma cholesterol concentrations. Overexpression of SR-A in hepatocytes, under the control of the transferrin promoter, also decreased plasma cholesterol concentrations by reducing apoB-containing lipoproteins (45). Unlike this study, we did not observe an increase in HDL-C concentrations in mice overexpressing SR-A. In agreement with SR-A affecting plasma cholesterol concentrations, apoE$^{-/-}$ mice that are also deficient in SR-A have plasma cholesterol concentrations that are 46% higher than those of mice that are only apoE deficient (8). This study did not demonstrate whether the increase was attributable to a change in a specific lipoprotein fraction. Further studies will be required to determine the mechanism of the plasma cholesterol lowering effect caused by SR-A overexpression.

In agreement with the premise that SR-A promotes atherogenesis, the initial report of SR-A deficient mice in the atherosclerosis susceptible background of apoE$^{-/-}$ reported a ~60% decrease in the size of lesions in the aortic root (8). However, subsequent studies using both SR-A deficiency and transgenic mice have not provided consistent results on the effect of this receptor on atherosclerosis, as recently reviewed (1). In the present study, we have restricted the expression of the transgene to macrophages through a combination of a cell-specific promoter and bone marrow transplantation. In the LDLr$^{-/-}$ mice, expression of this transgene clearly produced a decrease in the extent of lesion formation both in the aortic root and arch. A recent publication used a yeast artificial chromosome to create SR-A overexpressing mice and demonstrated that there was no effect on the development of atherosclerotic lesions in chimeric mice on an LDLr$^{-/-}$ background. In this publication, the transgene was under the control of the natural promoter for SR-A (13). Therefore, the lack of effect, compared with the present study, may be due to cytokines present in lesions that could have downregulated SR-A locally, even though the overexpression could be detected in cultured peritoneal macrophages.

Recent studies have used SR-A$^{-/-}$ bone marrow cell transfer to manipulate receptor activity and determine its effect on atherogenesis. In contrast to the results of the present study, Babaev et al. (11) demonstrated that transplantation of bone cells from the SR-A$^{-/-}$ mice did not have an effect on plasma cholesterol concentrations, but reduced atherosclerosis in the aortic root. This difference

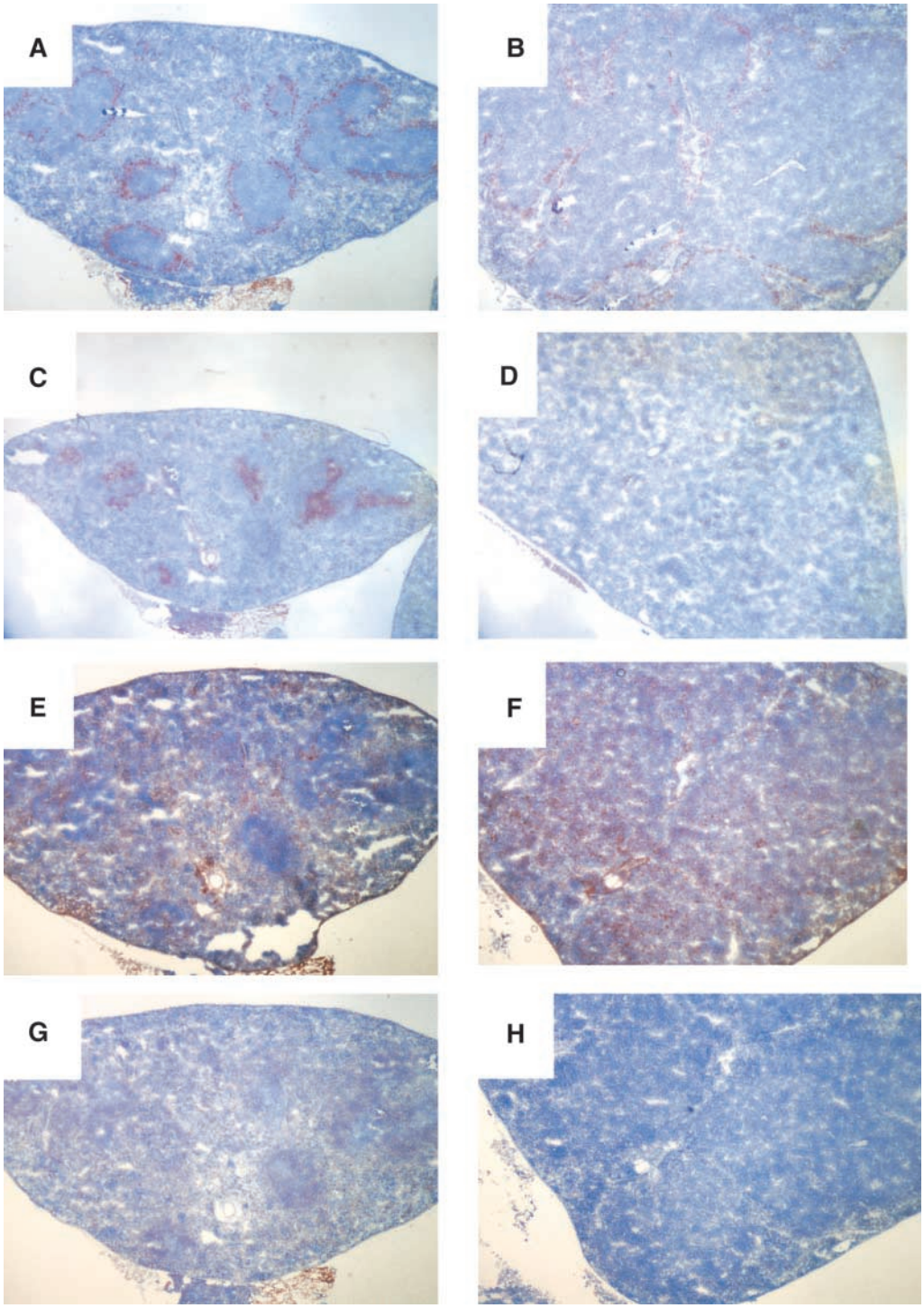

Fig. 6. Immunocytochemical characterization of spleens from LDLr$^{-/-}$ mice transplanted with bone marrow from non-transgenic (A, C, E, G) or transgenic littermates (B, D, F, H). Tissue sections were immunostained with: A, B: an antimouse MARCO monoclonal antibody; C–F: a monoclonal antimouse Thy-1.2 antibody; G and H: a rabbit antisera to mouse macrophages. Magnification is 200×.

may relate to a gender-specific effect with females used in the present study versus males in the study of Babaev et al. (11). Also, our donor mice were a pure C57BL/6 background versus a six times backcross. Van Eck et al. (12) have also used bone marrow cell transfer from SR-A transgenic mice to female apoE$^{-/-}$ mice. The repopulation by SR-A transgenic bone marrow cells decreased plasma cholesterol concentrations but did not have an effect on the extent of lesion formation in the aortic root. Therefore, further studies need to define the basis for the variety of responses that have been observed by macrophage-specific regulation of SR-A.

The effect of SR-A on atherogenesis most commonly focuses on the ability of the receptor to internalize modified

lipoproteins. However, SR-A has other biological properties that could impact the atherogenic process. One of these recently defined properties is adhesion to substrata and lymphocytes (2–5). This adhesive property may be particularly prominent in regions of damaged extracellular matrix (4), as would be expected to occur during inflammatory processes within atherosclerotic lesions. Adhesion of macrophages to glycated collagen type IV through an SR-A mechanism ablates the accumulation of AcLDL (3). Therefore, the effect of SR-A on macrophage uptake of modified lipoproteins may be modulated in vivo as a consequence of bSR-A-mediated adhesion. Furthermore, it is possible that the metabolism of modified lipoproteins may be an effect that is demonstrable in cultured cells, but does not have relevance in vivo. This would be analogous to the effects of SR-A on apoptosis in cultured thymocytes, which do not occur in SR-A deficient mice (46, 47). Also, while deficiency of SR-A markedly diminishes the ability of cultured macrophages to recognize modified LDL, it has no effect on its metabolism in vivo (48, 49). This may be due to the redundancy of receptors, such as CD36 and macrosialin, which recognize modified forms of lipoproteins (50, 51).

Another recently recognized property of SR-A is its ability to interact with intracellular signaling pathways (6, 7, 52). One consequence of this intracellular signaling is an elaboration of urokinase type plasminogen activator that degrades extracellular matrix (6, 53). Given the observation that plasminogen deficiency greatly increases the extent of atherosclerosis in apoE$^{-/-}$ mice (54), stimulation of this pathway by SR-A could contribute to a decrease in atherosclerosis.

Associated with the reduction in atherosclerosis, overexpression of SR-A also invoked changes in the spleen. Increased spleen size and altered morphology in the lyso-bSRA repopulated animals was presumably related to the hyperlipidemic state of the lyso-bSR-A $\times$ LDLr$^{-/-}$ chimeric mice, since no changes were observed in the transgenic donor mice. SR-A has been previously demonstrated to mediate integrin-independent adhesion of macrophages to the red pulp and the marginal zone of the spleen (17). Therefore, part of the increased size may relate to the increased presence of adherent macrophages. However, the mechanism underlying the effect on the spleen is undefined at present.

In summary, we have demonstrated significant effects of macrophage-specific overexpression of SR-A on the development of atherosclerotic lesions and characteristics of spleen tissue in LDLr$^{-/-}$ mice. The mechanism by which overexpression of SR-A influences these parameters remains to be determined.

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