Macrophage-specific expression of class A scavenger receptors enhances granuloma formation in the absence of increased lipid deposition

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Abstract **Class A scavenger receptors (SR-A) have several proposed functions that could impact atherosclerosis and inflammatory processes. To define the function of SR-A in vivo, we created C57BL/6 transgenic mice that expressed bovine SR-A under the control of the restricted macrophage promoter, lysozyme (lyso-bSR-A). bSR-A mRNA was present in cultured peritoneal macrophages of transgenic mice and tissues that contain significant macrophages including spleen, lung, and ileum. Functional overexpression of SR-A was demonstrated in peritoneal macrophages both by augmented cholesterol ester deposition in response to AcLDL and enhanced adhesion in transgenic mice compared with nontransgenic littermates. To determine whether macrophage-specific expression of bSR-A regulated inflammatory responses, granulomas were generated by subcutaneous injection of carrageenan. Granuloma size was significantly increased in lyso-bSR-A transgenic mice compared with wild-type littermates [421 ± 51 mg (n = 11) vs. 127 ± 22 mg (n = 10), $P < 0.001$]. However, the larger granulomas in lyso-bSR-A transgenic mice were only associated with an increase in unesterified cholesterol, and not cholesterol esters. Furthermore, granulomas from transgenic mice had an increase in the number of macrophages within the tissue. Therefore, macrophage expression of bSR-A increased presence of this cell type in granulomas without enhancing the deposition of cholesterol esters, consistent with a role of the adhesive property of the protein.**—Daugherty, A., N. Kosswig, J. A. Cornicelli, S. C. Whitman, S. Wolle, and D. L. Rateri. **Macrophage-specific expression of class A scavenger receptors enhances granuloma formation in the absence of increased lipid deposition.** ***J. Lipid Res.*** **2001.** 42: **1049–1055.**

Supplementary key words cell-specific promoter • cholesterol • lysozyme • inflammation

Class A scavenger receptors (SR-A) have been proposed to be involved in several pathophysiological processes including atherosclerosis and host defense. Many of these properties were initially inferred from cell culture studies, but have been substantiated with the development of mice that are SR-A deficient (1). The array of potential biological properties of SR-A may be due partly to the promiscuous ligand binding properties. Known ligands include modified lipoproteins, polynucleotides, polysaccharides, phospholipids, and bacterial products (2). In addition, SR-A also has adhesive properties for binding to serum-coated surfaces (3), modified extracellular matrix (4), and B lymphocytes (5). Therefore, SR-A has the potential to exert a variety of biological effects both through internalization of ligands and promoting cell adhesion.

SR-A activity was originally described in macrophages (6). Subsequent studies have substantiated the presence of the protein in this cell type, although its expression varies as a function of phenotype (7) and the presence of its numerous regulators (2). SR-A has been described both in vitro and in vivo in smooth muscle cells (8–10), and the rabbit cDNA was cloned from this cell type (11). SR-A activity is present in many endothelial beds and the presence of the protein has been described in hepatic sinusoidal (12), post-capillary venular (13), and aortic endothelium (14). Therefore, the effects of SR-A deficiency that have been described in genetically deficient mice (1) may be attributable to the absence of this receptor at several cell types.

Because SR-A has the potential to be expressed by many cell types, the biological effects of SR-A deficiency in genetically engineered mice may be attributable to a number of complex mechanisms involving interactions between several cell types. To determine the effects of macrophage-specific expression of SR-A on the many biological properties implied for this receptor, transgenic mice can be generated using promoters that are restricted to this cell lineage. The SR-A promoter itself has been sug-

Abbreviations: AcLDL, acetylated LDL; lyso-bSR-A, bovine SR-A expressed under the control of lysozyme; MPM, mouse peritoneal macrophages; SR-A, class A scavenger receptor.

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gested to be macrophage specific (15), although there is limited evidence to support this notion (16). More recently, the macrosialin promoter has been shown to be highly active in cultured macrophages (17), although its utility in vivo is uncertain. The chicken lysozyme minigene construct, on the other hand, has been extensively characterized and shown to be macrophage specific (18, 19). This promoter has been used to drive macrophage-specific expression of 15-lipoxygenase in rabbits and influence the atherogenic process (20, 21).

The purpose of this study was to develop transgenic mice that overexpress bovine SR-A (bSR-A) in a macrophage-specific manner using a chicken lysozyme promoter (lyso). Our initial studies demonstrated enhanced recognition of the SR-A ligand, acetylated LDL (AcLDL) (22), by cultured peritoneal macrophages from these transgenic mice (23). These cells also exhibited increased spreading, consistent with the adhesion function of SR-A (1, 3). Having demonstrated that these mice exhibited functional characteristics of SR-A overexpression, we determined that overexpression of SR-A significantly increased the development of carrageenan-induced granulomas. This effect was not due to enhanced lipid deposition but, rather, was due to increased number of macrophages.

MATERIALS AND METHODS

Mice and diets

Transgenic mice were generated by the University of Michigan's Transgenic Core Facility directly in the C57BL/6 strain. The resulting transgenic mice were bred to C57BL/6 mice obtained from the National Cancer Institute (Frederick, MD). All mice were fed either a standard laboratory diet or one enriched in cholesterol (1.25%), saturated fat (21%), and cholate (0.5%) as described by Paigen et al. (24). Water was available ad libitum. Mice were greater than 9 months of age at the time they were used for studies. All studies involving animals had the prior approval of the University of Kentucky Institutional Animal Care and Use Committee.

Construction of the bovine SR minigene and generation of transgenic animals

The full-length bovine SR-A cDNA was prepared as previously described (25) and was inserted into a chicken lysozyme genomic DNA construct [a generous gift from Dr. Albrecht Sippel; (19)]. This minigene construct contained 11.5 kb 5′- and 5.5 kb 3′-flanking sequences, and has been used previously to drive macrophage-specific gene expression (20). It was provided to the University of Michigan Transgenic Core Facility for injection into fertilized one-cell embryos from C57BL/6 mice. Potential founder animals were screened by Southern blot analysis, and one animal that incorporated the transgene into the germ line was bred with nontransgenic C57BL/6 mice.

PCR screen for bovine SR-A DNA

DNA was isolated from tail clips using a commercially available kit (Qiagen; Valencia, CA). The following primers were used: 5′-ATGCGACAGTGGGATGACTTTC-3′ and 5′-CAACCGT GCAATTCTTCGTTTC-3′. These primers yielded a 255-bp fragment corresponding to nt 22–277 of bSR-A. The parameters for the thermocycle reaction were 95°C for 4 min; 35 cycles of 96°C for 1 min, 56°C for 30 s, and 72°C for 30 s; one cycle of 72°C for 15 min. Using these conditions, no reaction products were observed in wild-type strain-matched mice.

Extraction and detection of transgene mRNA

Selected tissues were rapidly isolated, flash frozen in liquid nitrogen, and stored at −70°C. Total RNA was isolated using the SV Total Isolation System (Promega, Madison, WI). Transgenic mRNA was detected with the Access RT-PCR system (Promega) using the primers described above. Controls were performed in the absence of reverse transcriptase.

Lipoprotein isolation and modification

LDL (density 1.019–1.063 g/ml) was prepared from fresh human plasma by differential ultracentrifugation as described previously (26, 27). AcLDL was prepared as described by Basu et al. (23).

Harvest of mouse peritoneal macrophages

Mice were anesthetized with methoxyflurane and exsanguinated. Macrophages were harvested via peritoneal lavage using sterile saline (5 ml). Cells were resuspended in DMEM (catalog no. 11965-084; Gibco/BRL, Grand Island, NY) containing heat-inactivated fetal bovine serum (10% v/v; catalog no. 16140-071; Gibco/BRL), penicillin, and streptomyocin, and plated in 12-well plastic tissue culture plates (Costar, Corning, NY). Cells were incubated at 37°C with 5% CO_2 overnight prior to performing assays.

Quantification of the size of cultured macrophages

Peritoneal macrophages were plated in 8-well LabTek slides (Nalge Nunc International, Rochester, NY) at a density of 1×10^5 per well. Cells were fixed by incubation with paraformaldehyde (4%) after incubation for intervals up to 24 h. Macrophages were immunostained with a rabbit antiserum to mouse macrophages (Accurate, Westbury, NY), and area of cells was quantified as described previously (28).

Cholesterol ester synthesis

Cholesterol ester synthesis in cultured peritoneal macrophages was determined as described previously (29). Briefly, cells were incubated with the stated concentrations of AcLDL protein/ml and [^{3}H]oleate (0.1 mM) for 5 h at 37°C in tissue culture media without serum. The media was removed, and the cells were washed and subjected to lipid extraction using hexane–isopropanol (3:2, v/v). The lipid extracts were evaporated to dryness with N_2, redissolved in $CHCl_3$–MeOH (2:1, v/v), and separated by thin-layer chromatography. The cholesterol ester band was isolated and radioactivity content was quantified.

Serum cholesterol concentrations

Serum cholesterol concentrations were determined using commercial enzymatic assay kits (Wako Chemical Co., Richmond, VA).

Granuloma formation

Mice were anesthetized with methoxyflurane. An air pouch was formed by injecting 1 ml of air into the subcutaneous space. A solution of carrageenan (200 μl of 1% solution in sterile water) was injected into this location within 1 min. Granulomas were removed from the subcutaneous pouch 7 days later and weighed. Half the tissue was frozen in OCT for histological analysis, whereas the other half was utilized for tissue lipid analysis.

Characterization of granulomas

Neutral lipids were visualized using Oil Red O. Frozen sections were fixed in 4% paraformaldehyde, equilibrated in 60% propanol, stained with Oil Red O (0.25% solution) for 20 min, and destained in 60% propanol.

To quantify the sterol content of the granulomas, weighed tissues were extracted using the method described by Folch as detailed previously (30). Extracts were resolublized in a buffer containing Triton-X100 as described previously (31). Cholesterol

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and cholesterol esters were quantified in aliquots of the extract using commercial enzymatic kits (Wako).

Immunocytochemistry was performed, as described previously (32), on frozen sections using Vector avidin-biotin complex Elite kits (Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized using the red chromagen, amino-ethyl carbazole (Biomeda, Foster City, CA). Macrophages were detected using a rabbit antiserum to mouse macrophages as described above. Nonimmune rabbit serum was used as a control for the antiserum.

Statistics

Data analyses were performed using SigmaStat 2.03 software (SPSS Inc., Chicago, IL). For each parameter, the mean and standard error of mean were calculated. Statistical analysis between groups was performed by Student's *t*-test after testing that the data complied with the constraints of parametric analysis. Chi-squared analysis with a Yates correction factor was used to analyze macrophage sizes. Values with $P < 0.05$ were considered to be statistically significant.

RESULTS

Creation and characterization of lyso-bSR-A transgenic mice

The transgenic animals showed no obvious phenotype and appeared healthy throughout their lifespan. Transgenic mice were bred to wild-type strain equivalent mice and the offspring were ~50% positive for the presence of the bSR-A cDNA. Expression of bSR-A was detected by RT-PCR in transgenic-positive peritoneal macrophages, but not in negative littermate mice. The highest expression of bSR-A in the tissues surveyed was found in lung, peritoneal macrophages, and spleen (**Fig. 1**). There was no expression detected by RT-PCR in nontransgenic littermates, or when RNA from transgenic mice was used in the absence of reverse transcriptase (data not shown). Due to the lack of availability of an antibody to specifically detect the transgenic protein, we have not been able to determine whether the extent of protein expression is related to the level of expression of mRNA. Therefore, to demonstrate the presence of the transgene, we performed functional assays to provide evidence for expression of bSR-A.

SR-A was originally described as a receptor for AcLDL. Therefore, we quantified cholesterol esterification in cultured peritoneal macrophages in the presence and absence of AcLDL. Under basal conditions, there was little incorporation of [^{3}H]oleate into cholesterol esters in cells from either the transgenic or nontransgenic littermates. Although cholesterol ester deposition was increased in the presence of AcLDL, the stimulation was markedly augmented in macrophages from the transgenic mice (**Fig. 2**).

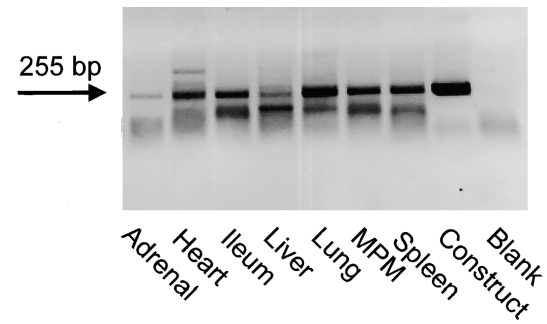

Fig. 1. Tissue and cell expression of transgene in lyso-bSR-A mice. Total RNA was isolated from the tissue and MPM, as indicated on the figure. RT-PCR was performed that generates a specific product of 255 bp.

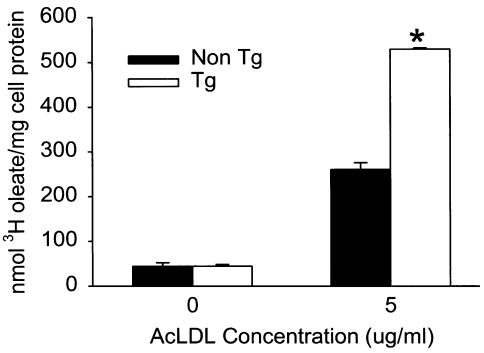

Fig. 2. Stimulation of cholesterol ester synthesis by AcLDL in the cultured peritoneal macrophages from nontransgenic (Non Tg; closed bar) and transgenic (open bar) littermates. Histobars represent the mean of six observations from two experiments, and bars represent the SEM. * $P < 0.001$.

SR-A has also been described as an adhesion molecule, which is based partly on the limited ability of peritoneal macrophages from SR-A-deficient animals to spread following plating (1, 3). To determine whether the converse occurred in peritoneal macrophages from lyso-bSR-A transgenic animals, cells were plated for selected intervals and immunostained. By visual inspection, cells from transgenic mice were larger than those from nontransgenic littermate controls after 3, 6, and 24 h of incubation. An example of cells at 24 h is shown in **Fig. 3A** and **B**. Quantification of the size of these cells demonstrates that macrophages from transgenic mice spread further than the cells from nontransgenic littermates. At 24 h, there was a significantly increased number of cells with an area of greater than 400 μm^2 ($P < 0.001$) in the macrophages cultured from transgenic mice compared with their nontransgenic littermates (Fig. 3C).

Formation of granulomas

Formation of granulomas was examined in transgenic and nontransgenic littermate mice fed a diet enriched in saturated fat, cholesterol, and cholate. Although this same diet has been used by others to generate a hyperlipidemic response (33), we did not observe a significant hyperlipidemic response. Furthermore, serum plasma cholesterol concentrations were not significantly different in the lyso-bSR-A transgenic animals (**Fig. 4**).

Subcutaneous injection of carrageenan resulted in the formation of granulomas that reached maximal size after 7 days. Based on these preliminary studies, we determined the effects of the presence of the transgene on the size and characteristics of granulomas. Lyso-bSR-A transgenic mice developed granulomas that were visually larger and had 3.3 times greater mass than those formed in the non-

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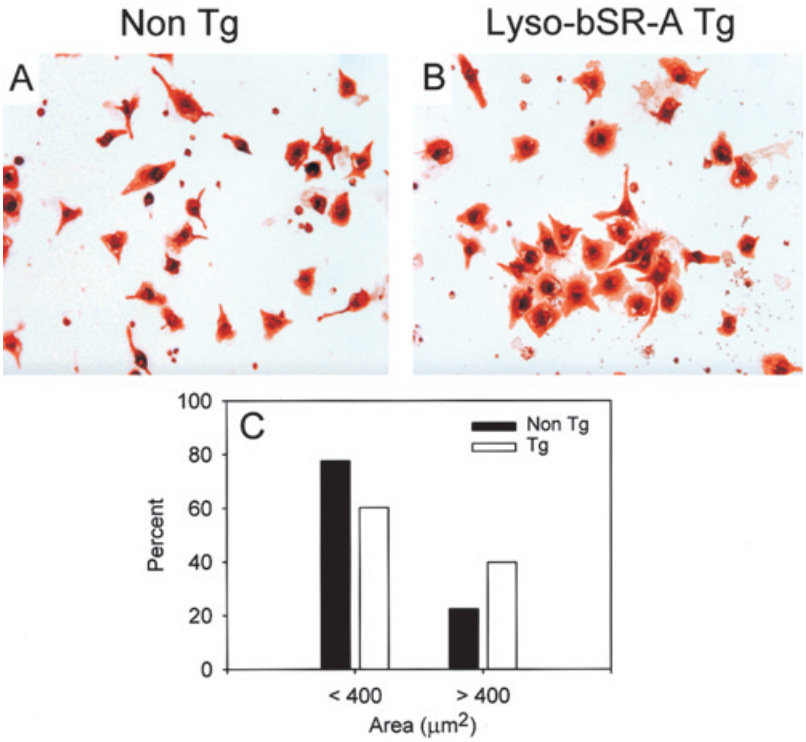

Fig. 3. Examples of cultured peritoneal macrophages from nontransgenic (A) and lyso-bSR-A transgenic (B) littermates. Macrophages were incubated for 24 h and quantified for surface area in nontransgenic (closed bar) and transgenic (open bar) littermates as described previously (28). C: Histobars represent the percentage of cells that are either above or below 400 μm^2 in surface area. ($P < 0.001$).

transgenic littermates (421 ± 51 mg vs. 127 ± 22 mg, $P = 0.001$; **Fig. 5**). The sterol content of the granulomas was analyzed as described in Materials and Methods. Although total cholesterol content was increased in the granulomas from lyso-bSR-A mice to a comparable extent with the increase in size of the tissue, the increase was attributable to an increase in unesterified cholesterol, with no increase in the esterified form (**Fig. 6**).

To provide more insight into the mechanisms of this increase in size of carrageenan granulomas generated by transgenic expression of bSR-A, the histological characteristics of the granulomas were examined. In agreement with the sterol measurements, there was very little neutral lipid stain in granulomas from either transgenic or nontransgenic littermates (**Fig. 7A**). Although there was considerable heterogeneity in the morphology of granuloma tissue, one consistent feature was a greater density of immunostainable macrophages in the granuloma tissue from the lyso-bSR-A transgenic mice (Fig. 7B).

DISCUSSION

In the present study, we describe the development of mice that express b-SR-A under the control of the chicken lysozyme promoter. This promoter construct has been reported previously to result in macrophage-specific expression of other transgenes including reporter proteins and 15-lipoxygenase (18, 34). In the present study, bSR-A mRNA was highly expressed in tissues containing macrophages including the spleen, lung, and ileum. We were unable to determine whether the expression of this gene was restricted to cells of macrophage lineage within these tissues due to our lack of an antibody that specifically recognizes the bovine form of SR-A (3). However, in isolated peritoneal macrophages, there was pronounced mRNA

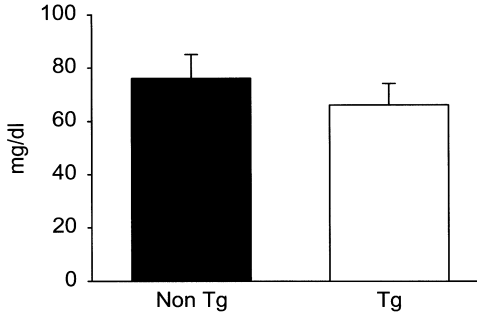

Fig. 4. Plasma cholesterol concentrations during feeding of a modified diet to nontransgenic (closed bar) and transgenic (open bar) littermates. Histobars represent the mean of 12 observations, and bars represent the SEM. The difference between the means is not statistically different.

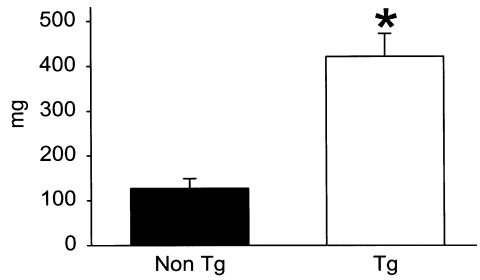

Fig. 5. Weight of granulomas formed 7 days after subcutaneous injection of carrageenan in nontransgenic (closed bar) and transgenic (open bar) littermates fed a high fat diet. Histobars represent the mean of 12 observations, and bars represent the SEM. * $P < 0.001$.

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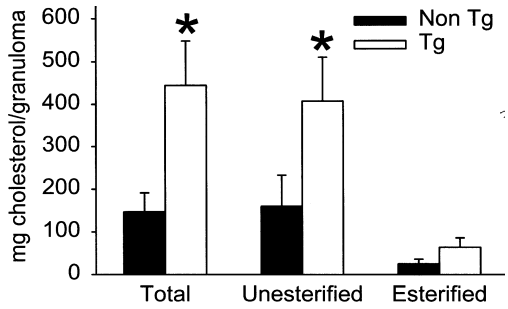

Fig. 6. Sterol content of granulomas described in Figure 5. The total and unesterified cholesterol content was determined as described in Materials and Methods in nontransgenic (closed bar) and transgenic (open bar) littermates fed a high fat diet. Histobars represent the mean of 12 observations, and bars represent the SEM. * $P < 0.01$.

expression of the transgene. In these cells, we were also able to perform functional assays to determine the biological activity of the transgene. In agreement with the detection of mRNA, there was an augmented esterification of cholesterol in response to AcLDL, a specific ligand for SR-A, in peritoneal macrophages from transgenic mice compared with nontransgenic littermates. Furthermore, there was increased spreading of cultured macrophages from lyso-bSR-A transgenic mice, consistent with increased adhesion (1, 3).

Having demonstrated that the presence of the transgene led to increased SR-A activity, we determined whether this influenced the development of granolumas induced by subcutaneous administration of carrageenan. Carrageenan-induced granulomas have been used as a model of foam cells to elucidate mechanisms of atherosclerosis (35, 36). Subcutaneous placement of carrageenan in cholesterol-fed and Watanabe heritable hyperlipidemic rabbits resulted in the formation of cholesterol ester-rich granulomas (35, 36). This lipid engorgement in these rabbits was correlated to the high plasma cholesterol concentrations that are attained in these rabbits. In contrast, in the present study, the carrageenan granulomas did not have a predominance of cholesterol in its ester form in either transgenic or nontransgenic littermates. The lack of neutral lipid accumulation is in agreement with the characteristics of granulomas induced in normolipidemic rabbits (37). Contrary to the reports of others, we were unable to induce pronounced hyperlipidemia in C57BL/6 mice fed a diet enriched in saturated fat, cholate, and cholesterol. This has been a consistent finding with many studies conducted in our laboratory, despite using a diet and animal source that is consistent with others (38). Under these conditions, the macrophage-specific expression of bSR-A did not change the serum concentrations of cholesterol, as has been noted in hyperlipidemic states (25, 39).

Transgenic mice that expressed the bSR-A under the control of the chicken lysozyme promoter developed larger granulomas than their nontransgenic littermates. Based on the quantification of lipids and histological staining, the small content of cholesterol esters and neutral lipids demonstrate that the increased size is not due to SR-A promoting foam cells formation as invoked by others

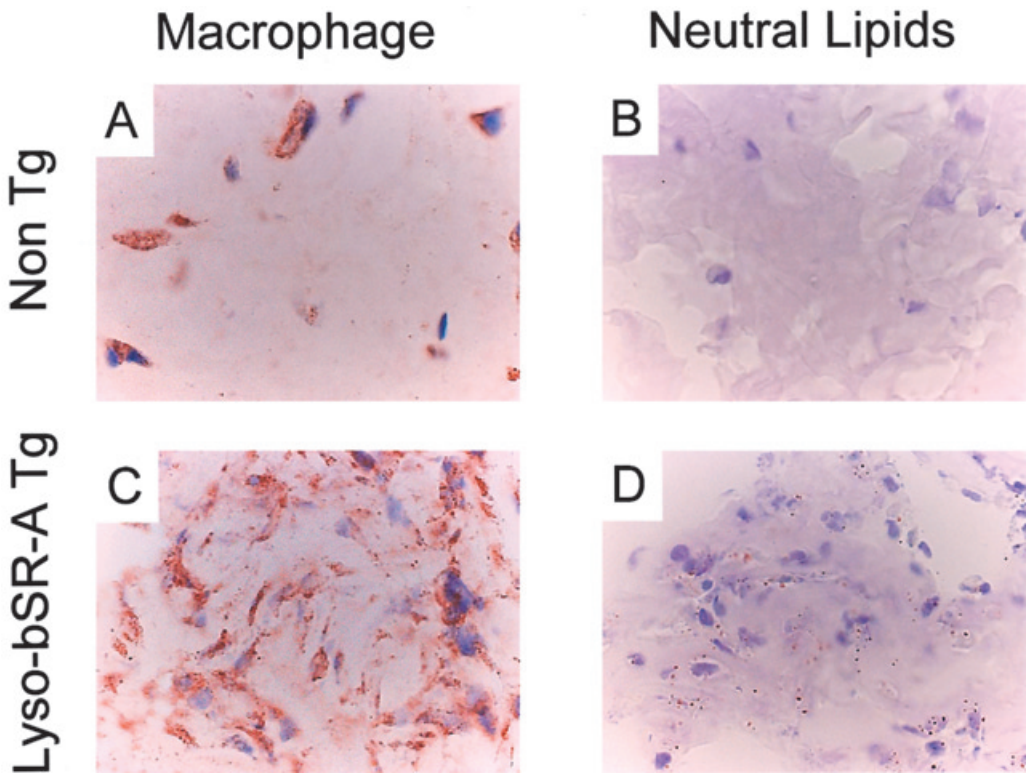

Fig. 7. Characteristics of carrageenan-induced granulomas from nontransgenic (A, B) and lyso-bSR-A transgenic (C, D) littermates stained for macrophages (A, C) or neutral lipids (B, D).

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(36). Because there was an increased cellularity of the granulomas, we suggest that enhanced SR-A expression is functioning through adhesion. One mechanism of increased adhesion may be due to the residual presence of carrageenan, which is a ligand for SR-A (40). Another possible explanation may be enhanced interactions with the extracellular matrix of the granuloma as has been described recently (3, 4, 41). Unlike the domain of SR-A that mediates lipoprotein binding (42), the domain responsible for adhesion has not been determined. If the domains of lipoprotein binding and adhesion differ, it may be possible to generate transgenic mice that lack these specific properties in order to assess their contribution to granuloma formation. However, at present, is it not possible to define the mechanism that underlies the increased granuloma formation.

The effect of SR-A deficiency has been examined on the development of *Corynebacterium parvum*-induced liver granulomas (43). Lack of SR-A attenuated the increase in *C. parvum*-induced liver weight seen in wild-type animals, with changes in the maximal number and diameters of granulomas formed. In this model, the reduced uptake of *C. parvum* by SR-A−/− macrophages probably accounted for the differences in granuloma formation. Therefore, the mechanism of changes in granuloma formation in SR-A−/− mice promoted by *C. parvum* injection is likely to differ from that observed in the carrageenan-induced granulomas used in the present study.

SR-A was originally thought to promote atherosclerosis through mediating foam cell formation. Results of studies using SR-A−/− mice that are also deficient in either apoE or LDL receptors are consistent with a proatherogenic role of SR-A (1, 44). However, the summary of all studies using SR-A-deficient or overexpressing mice is that SR-A may be pro- or antiatherogenic depending on the stimulus used to invoke the disease (2). There is no clear explanation why SR-A should produce these diverse effects. The existence of transgenic mice that overexpress the receptor in specific cell types on identical genetic backgrounds as those commonly used atherosclerosis susceptible strains of mice will facilitate the elucidation of these diverse effects of SR-A on lesion formation.

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