Macrophage-Specific Expression of Mannose-Binding Lectin Controls Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice

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Background—With consideration of the central role of the innate immune system in atherogenesis and mannose-binding lectin (MBL) as an innate regulator of immunity, the role of MBL in experimental and human atherosclerosis was assessed.

Methods and Results—With the use of immunohistochemistry and polymerase chain reaction, deposition and gene expression of MBL-A and -C were assessed in murine atherosclerosis from mice deficient for the low-density lipoprotein receptor ($LDLR^{-/-}$) after 10 or 18 weeks of high-fat feeding. MBL was present and was produced in 10-week-old lesions, whereas deposition and gene expression were minimal after 18 weeks of high-fat feeding and absent in healthy vasculature. Interestingly, deposition of MBL-A and -C differed: MBL-A predominantly localized in upper medial layers, whereas MBL-C was found in and around intimal macrophages. To further study the role of local MBL production by monocytic cells in atherosclerosis, $LDLR^{-/-}$ mice with MBL-A and $-C^{-/-}$ monocytic cells were construed by bone marrow transplantation. Mice carrying MBL-A and -C double deficient macrophages had increased (30%) atherosclerotic lesions compared with wild-type controls ($P=0.015$) after 10 weeks of high-fat diet. Subsequently, analysis of MBL deposition and gene expression in advanced human atherosclerotic lesions revealed the presence of MBL protein in ruptured but not stable atherosclerotic lesions. Putatively in agreement with murine data, no MBL gene expression could be detected in advanced human atherosclerotic lesions.

Conclusions—These results are the first to show that MBL is abundantly present and locally produced during early atherogenesis. Local MBL expression, by myeloid cells, is shown to critically control development of atherosclerotic lesions. **(*Circulation*. 2009;119:2188-2195.)**

Key Words: arteriosclerosis ■ cardiovascular diseases ■ immunology ■ molecular biology

Recently, the concept that different elements of the innate immune system mediate atherosclerosis has gained significance.[1,2] In this respect, it is not surprising that complement components were detected in atherosclerotic lesions and shown to be of importance during atherogenesis from the earliest stages of plaque formation to the progression of advanced lesions.[3,4]

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Mannose-binding lectin (MBL) is a C-type or Ca^{2+}-dependent lectin that can act as an opsonin and can activate complement through its own and evolutionary conserved lectin pathway.[5] There is considerable variation in activating properties and plasma levels between individuals. These variations are caused by 3 first exon mutations (at codons 52, 54, and 57) and polymorphisms in the promoter region (at positions −550 [H/L] and −221 [X/Y]) of the structural gene (*mbl2*).[6] Studies on the development of vascular diseases, including atherosclerosis in MBL-sufficient and -insufficient human subjects, suggested that the MBL C-type lectin influences vascular disease.[7–13] Frequently, low MBL levels in MBL-deficient patients have been associated with an earlier disease onset or a more progressive disease course compared with their MBL-sufficient counterparts. However, opposing contributions by MBL to atherogenesis have been proposed as well. MBL-mediated modulation of *Chlamydia pneumoniae* infection, differences in complement activation, deficiencies in MBL-opsonizing capacities, and unknown sex-

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related differences may explain frequently observed associations between cardiovascular disease and *mbl2* gene variations.[7,10,14,15]

The long-existing tenet that merely centrally produced complement components drive inflammatory reactions has recently been challenged.[16–19] Locally synthesized complement proteins such as C1q and C3 greatly determine the innate immune response, contributing to, for example, ischemia/reperfusion-induced tissue damage.[19] First reports suggest similar effects of locally produced complement components over the course of not only ischemia/reperfusion injury but also during development of atherosclerosis and neointima formation after arterial injury.[17,18,20,21]

The ostensible contradiction that remains regarding either a proatherogenic or antiatherogenic effect of MBL in atherosclerosis led us to investigate basic MBL characteristics over the course of atherogenesis in atherosclerosis-prone mice and human atherosclerotic lesions. Our data demonstrate the presence and distribution of MBL in experimental and human atherosclerotic lesions and provide compelling evidence for a controlling role of local myeloid-derived MBL expression in atherosclerosis development. The importance of MBL expression by myeloid cells in atherosclerosis development creates new insights into the role of this versatile immune regulatory protein during atherogenesis.

Methods

For additional details, please see the online-only Data Supplement.

Mice and Preparation of Tissues

Mice were kept according to University of Maastricht animal facility regulations, and all experiments were approved by the local Animal Ethical Committee. Mice deficient for the low-density lipoprotein receptor ($LDLR^{-/-}$) on a C57BL/6J background, 10 weeks old and weighing 25 g, were purchased at Jackson Laboratories (Bar Harbor, Me). Hearts and aortic arches were excised and embedded in OCT compound and frozen on dry ice and subsequently stored at −70°C.

Immunohistochemical Staining

Immunohistochemical stainings were performed with the use of a monoclonal antibody to murine macrophages (FA-11), which was kindly provided by Dr S. Gordon (Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom); to murine smooth muscle actin (1A4), which was purchased from Sigma (St Louis, Mo); to MBL-A (8G6) and MBL-C (14D12), all kindly provided by Hycult Biotechnology (Hbt, Uden, Netherlands); and to human MBL (ATLAS Antibodies, Stockholm, Sweden). Sirius red staining was performed to visualize collagen, and toluidine staining was used to visualize lesion structure. Colocalization studies were conducted by staining serial sections.

Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from differently staged murine atherosclerotic lesions and healthy vascular tissue with the use of the SV Total RNA isolation system (Promega Corporation, Madison, Wis). DNA contamination was eliminated by treating isolates with RQ1 RNase-Free DNase (Promega). Total RNA was reverse-transcribed with oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, Calif). cDNA synthesized from murine liver-derived total RNA isolate served as positive control sample for both MBL-A and MBL-C. The amount of amplified product was measured by the Nano-drop absorption measurement. The polymerase chain reaction conditions and visualization were described earlier.[22]

Bone Marrow Transplantation

Forty 9-week-old female littermate $LDLR^{-/-}$ mice on a C57BL/6 background (Jackson Laboratories) were put in filter-top cages. The mice received acidified water supplemented with neomycin (100 mg/L) and polymyxin B sulfate (60 000 U/L) starting the week before bone marrow transplantation until 5 weeks after bone marrow transplantation. Bone marrow was transplanted 2 weeks after antibiotic treatment was ended. One day before the actual transplantation, the mice were irradiated with a lethal dose of 10-Gy radiation. The bone marrow of 5 female wild-type (WT) mice (C57BL/6 from Taconic, Germantown, NY) and 5 female transgenic MBL-A and -C double knockout mice (C57BL/6, kindly provided by Dr S. Thiel, Institute of Medical Microbiology and Immunology, Aarhus University, Aarhus, Denmark) was collected. Bone marrow cells were harvested by flushing of the murine femur. For transplantation, 20 mice received WT bone marrow (WT-Tx), and 20 mice received transgenic bone marrow (KO-Tx). Bone marrow cells (10^6) were injected into the tail vein of each mouse.

Blood and Chimerism Analysis

Seven weeks after transplantation, the mice were put on a high-fat diet for 10 weeks. Chimerism was analyzed as described before,[23] 7 weeks after transplantation and before start of high-fat feeding. After 10 weeks of high-fat diet, blood was collected from the mice after overnight fasting to determine plasma cholesterol and plasma triglyceride concentration. Plasma cholesterol and triglycerides were determined with the use of the CHOD_PAP enzymatic essay kit (Roche, Basel, Switzerland) and GPO-Trinder kit (Sigma), respectively, according to the manufacturer's guidelines. Peripheral blood leukocyte levels were analyzed after 5 weeks of high-fat feeding.

Atherosclerosis Assessment and Lesion Analysis

After 10 weeks of high-fat diet, the mice were killed. Atherosclerosis was analyzed as described before.[23]

Human Atherosclerotic Lesions

Atherosclerotic lesions (total n=17; stable [n=8] and ruptured [n=9]) were collected during surgical procedures (carotid eversion endarterectomy) from individuals suffering end-stage symptomatic occlusive vascular disease. Tissue specimens were fixated in formalin or immediately frozen in liquid nitrogen and stored until analysis at −80°C.

Statistical Analysis

Data are expressed as mean±SEM and were analyzed by unpaired 2-tailed Student *t* test. Data were analyzed with the use of Prism 4.01 for Windows (Graphpad Software, San Diego, Calif). A probability value <0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

Results

MBL Deposition in Developing Atherosclerotic Lesions

To assess the deposition of the murine MBL variants -A and -C, atherosclerotic lesions from $LDLR^{-/-}$ mice after 10 weeks of high-fat feeding were analyzed. The lesions displayed known characteristics with a considerable influx of macrophages into the subendothelial space (Figure 1A). Macrophages infiltrating the developing lesions were detected by macrophage-specific CD68 expression. Medial collagen filaments as well as the development of a thin fibrous cap covering the intima were shown by Sirius red staining (Figure 1B). Both murine MBL variants were widely present in atherosclerotic plaques at this stage after 10 weeks

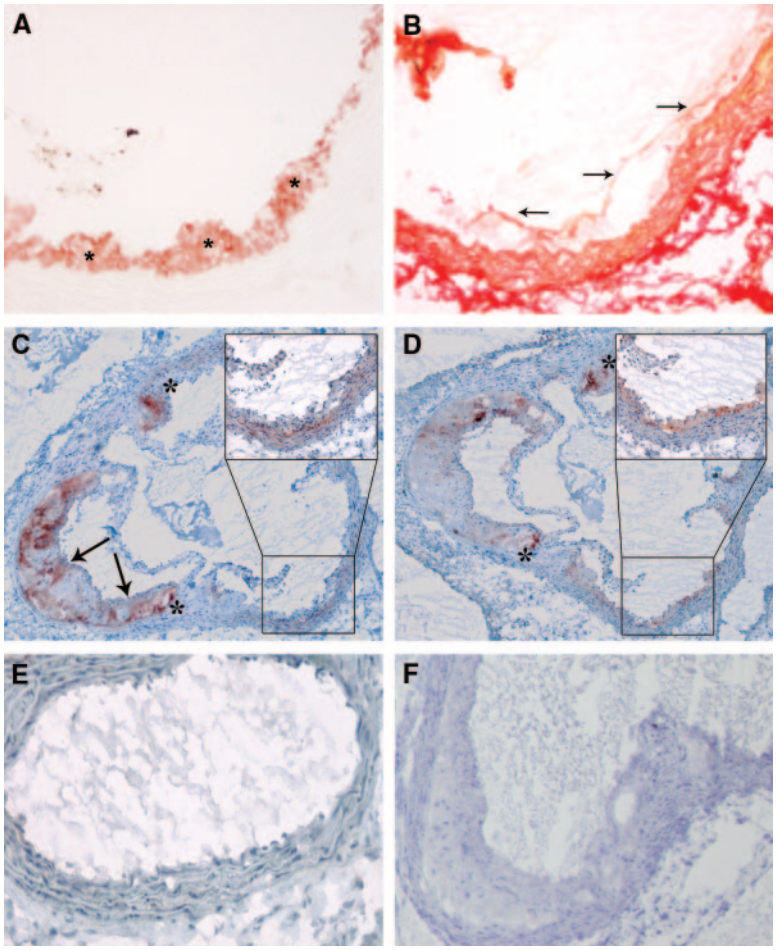

Figure 1. Characterization of MBL-A and MBL-C deposition in developing atherosclerotic lesions after 10 weeks of Western diet feeding. A, $CD68^+$ macrophages shown by 3-amino-9-ethylcarbazole staining (terra cotta red, *). B, Sirius red staining showing fibrous cap formation (arrows). C, MBL-A deposition. Arrow indicates the deposition of MBL-A along the fibrous cap. D, MBL-C deposition. E, MBL staining of healthy vascular tissue. F, Control staining, omitting the primary antibody (not shown) or using an isotype control antibody, remained negative. Magnification ×100 (A, B, E, and F), ×40 (C and D) (inserts ×100).

of Western diet feeding. Surprisingly, staining of the 2 MBL variants, with the use of serial sections, displayed different distribution patterns throughout 10-week lesions. MBL-A was frequently detected in the lower intimal layers directly above the media around the aortic sinus (Figure 1C). In addition, MBL-A was deposited on fibroblasts in the developing fibrous cap (Figure 1C, arrow). MBL-C, on the other hand, was detected solely in and around macrophages present in the intima (Figure 1D). Colocalization of MBL-A and -C occurred at sites of necrosis (as shown by the asterisk in Figure 1C and 1D). Healthy vasculature did not show staining for MBL-A (not shown) or MBL-C (Figure 1E).

MBL Deposition in Advanced Atherosclerotic Lesions

Next, the course of MBL deposition in atherosclerosis after prolonged high-fat feeding (18 weeks) was studied. General characteristics of advanced lesions were similar to those observed in younger lesions, containing a substantial number of invading macrophages (Figure 2A). A more developed fibrous cap covered sizable parts of the advanced lesion (Figure 2A, indicated by arrows). Together these structures composed the better part of the advanced atherosclerotic plaque. In these lesions, MBL-A and -C staining was generally decreased compared with the 10-week fat-fed mice. Moreover, the distinct distribution of MBL-A in the upper medial layers directly under the plaque as well as on the fibrous cap covering the lesion was decreased or was no longer observed (Figure 2B). The typical distribution observed for MBL-C in and around CD68-positive macrophages, seen in early atherosclerosis, was similarly diminished (Figure 2C). After 18 weeks of high-fat feeding, MBL-A and -C localized predominantly to sites of necrosis that were positively identified by a pathologist specifically trained in the field of murine pathology (Figure 2B and 2C, indicated by asterisk).

MBL-A and MBL-C Gene Expression in Atherosclerotic Lesions

To study local cellular MBL-A and -C production, MBL-A and -C gene expressions were assessed in total RNA, isolated from lesions obtained from $LDLR^{-/-}$ mice after various periods of high-fat feeding (10 or 18 weeks). Data were compared with data obtained from healthy vascular tissue. Figure 3 shows MBL-A and -C gene expression in 4 of 6 developing atherosclerotic lesions from $LDLR^{-/-}$ mice that were analyzed after 10 weeks of high-fat feeding. Surprisingly, only minimal MBL expression was detected in advanced lesions obtained from mice after 18 weeks of high-fat feeding. In agreement with the absence of cellular infiltrates in healthy vasculature, no MBL-A or -C mRNA was detectable. The data on MBL expression are supported by the immunohistochemical data that demonstrated abundant MBL presence in developing lesions, whereas only small amounts

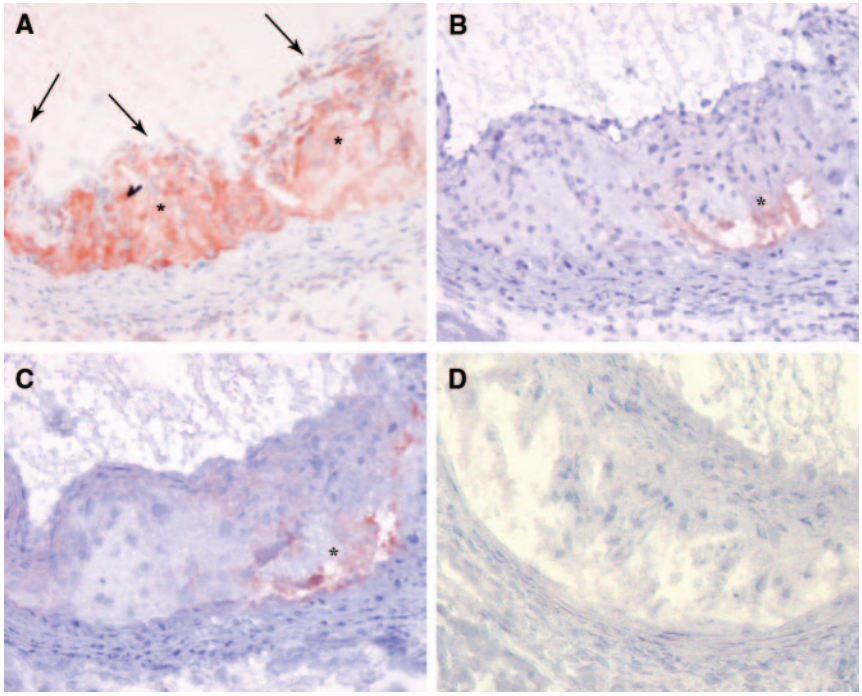

Figure 2. Characterization of MBL-A and MBL-C deposition in developing atherosclerotic lesions after 18 weeks of Western diet feeding. A, $CD68^+$ inflammatory cells in advanced atherosclerotic lesions (*). Fibrous cap covering the lesion (arrows) is shown. B, MBL-A deposition observed in necrotic core (*). C, MBL-C deposition observed in necrotic core (*). D, Control staining, omitting the primary antibody (not shown) or using an isotype control antibody, remained negative. Magnification ×100.

of MBL were detected in advanced atherosclerotic lesions, and no MBL was seen in healthy vascular tissue. Our reverse transcription polymerase chain reaction data suggest local MBL synthesis during early atherogenesis.

Bone Marrow Transplantation

Considering the distribution of MBL and the MBL gene expression in developing atherosclerotic lesions, we hypothesized that macrophages might be a local source of MBL. Earlier, Seyfarth et al[24] demonstrated *mbl2* gene expression in differentiated and lipopolysaccharide-treated THP-1 cells, a human monocyte cell line. Studies performed at our laboratory confirmed these findings (unpublished data). To study the significance of macrophage-derived MBL transcription in atherosclerosis, a bone marrow transplantation was performed with the use of 2 groups of 20 atherosclerosis-prone mice (female, $LDLR^{-/-}$) as recipients and WT or MBL-A and $-C^{-/-}$ donors. At the start of the high-fat feeding period, chimerism was similar in both groups (Table 1). At the end of the high-fat feeding period (10 weeks), total cholesterol and triglyceride plasma concentrations were statistically similar between groups (Table 1; $P=0.16$ and $P=0.77$, respectively). Total T-cell, B-cell, monocyte/macrophage, and granulocyte cell counts were similar between groups 11 weeks after bone marrow transplantation (Table 1). Interestingly, measurement of the total lesion area revealed a 30% increase in plaque size in $LDLR^{-/-}$ mice transplanted with MBL-A and $-C^{-/-}$ bone marrow (KO-Tx) after 10 weeks of high-fat feeding compared with $LDLR^{-/-}$ mice transplanted with WT bone marrow (WT-Tx) as the controls (Figure 4; $*P=0.015$). Figure 4 demonstrates microphotographs of representative atherosclerotic lesions from WT-Tx (Figure 4B) and KO-Tx (Figure 4C) mice. These data strongly suggest a determinative role for locally expressed MBL in the development of atherosclerosis.

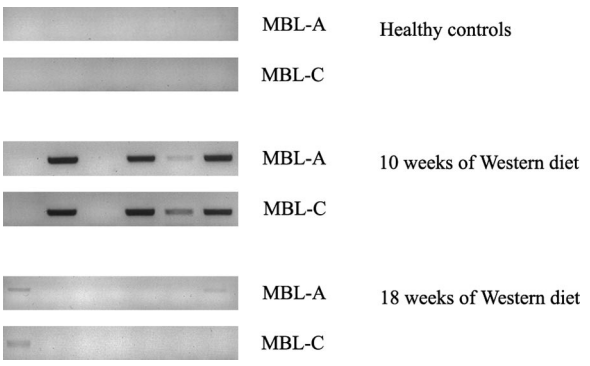

Figure 3. MBL synthesis was observed in 4 of 6 developing atherosclerotic lesions from $LDLR^{-/-}$ mice in response to 10 weeks of high-fat feeding (n=6). Remarkably, MBL synthesis was strongly reduced in atherosclerotic lesions from $LDLR^{-/-}$ mice after 18 weeks of high-fat feeding (n=7). No MBL synthesis was shown in healthy vasculature derived from $LDLR^{-/-}$ mice after 10 weeks of normal chow feeding (n=5).

Immunohistochemical staining for MBL-A and -C demonstrated reduced levels of both murine MBL variants in KO-Tx

Table 1. Chimerism, Total Cholesterol, Triglycerides, and Peripheral Blood Leukocytes in $LDLR^{-/-}$ Mice Transplanted With WT or MBL-$A^{-/-}$ and -$C^{-/-}$ Donor Bone Marrow*

	WT-Tx	KO-Tx	*P*
Chimerism, %	97.7±1.2	99.1±0.9	0.28
Total cholesterol, mmol/L	31.5±1.7	35.3±2.0	0.16
Triglycerides, mmol/L	1.2±0.2	1.2±0.1	0.77
T cells, %	14.7±1.1	16.2±0.9	0.32
B cells, %	54.9±2.0	56.7±1.5	0.47
Monocytes, %	6.5±0.3	5.8±0.4	0.22
Granulocytes, %	4.2±0.5	4.4±0.2	0.73

*Percentages given of total white blood cell count.

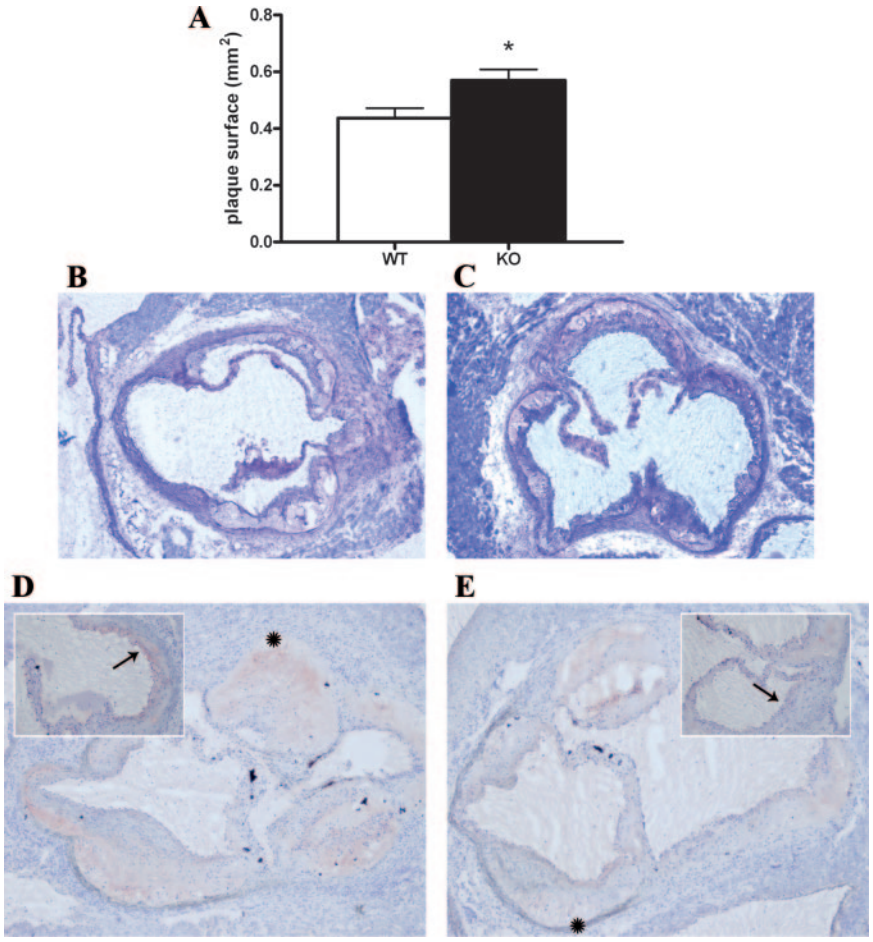

Figure 4. Development of atherosclerosis in WT-Tx and KO-Tx mice after 10 weeks of high-fat feeding. A, Increased lesion size (30%, $P=0.015$) was observed in KO-Tx animals compared with their WT-Tx controls after 10 weeks of high-fat feeding. B, Representative example of WT-Tx lesion (×40). C, Representative example of KO-Tx lesion (×40). D, MBL-A and MBL-C (insert) deposition in WT-Tx atherosclerotic lesions. MBL-C was detected mainly around intimal macrophages (arrow). E, MBL-A and MBL-C (insert) deposition were generally reduced in KO-Tx atherosclerotic lesions (*). MBL-C did not display its characteristic deposition around intimal macrophages in KO-TX lesions (arrow). Magnification ×40 (inserts ×100).

atherosclerotic lesions compared with WT-Tx controls (asterisk, Figure 4D and Figure 4E). In KO-Tx lesions, MBL-C did not display its typical distribution around intimal macrophages (WT-Tx in Figure 4D and KO-Tx in Figure 4E inserts). In KO-Tx, MBL was predominantly present around areas of necrosis, thereby resembling MBL distribution observed in 18-week lesions, in which no local MBL-A and -C gene expressions were observed, indicating the plasma origin at this stage.

Human Atherosclerosis

Human atherosclerotic lesions were obtained during elective surgical procedures. The collected lesions were divided into 2 distinct groups, stable (n=8) and ruptured (n=9), as assessed by a skilled pathologist on a hematoxylin-eosin staining (data not shown). Paraffin sections stained for human MBL deposition showed, interestingly, MBL deposition in ruptured lesions (Figure 5). MBL deposits were located within the enlarged intima, along ill-defined necrotic segments of the atherosclerotic plaque. Cholesterol crystals (indicated by an asterisk in Figure 5), frequently observed within intimal lesions, did not colocalize with MBL deposits. In stable lesions, no human MBL staining was observed (Figure 5, insert). To determine the origin of human MBL in ruptured lesions, quantitative polymerase chain reaction analysis was performed on cDNA from total RNA isolates of both stable and ruptured atherosclerotic lesions. Interestingly, human MBL gene expression was observed neither in ruptured nor in stable end-stage human atherosclerotic lesions (Table 2). Similar to the murine data, these data indicate that MBL deposition detected in advanced stages of atherosclerosis might well be plasma derived.

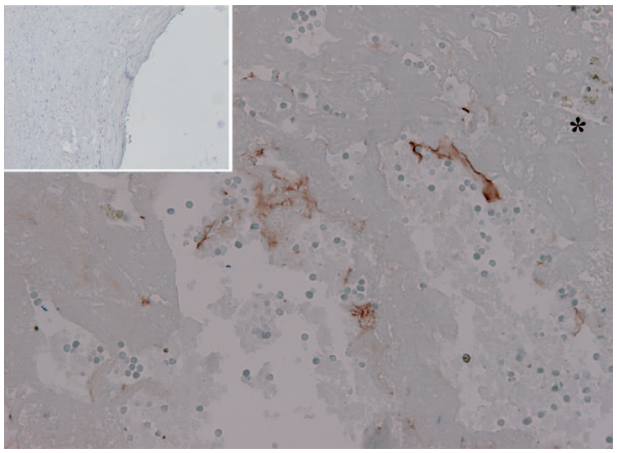

Figure 5. Human MBL (3-amino-9-ethylcarbazole staining, terra cotta red) was detected by immunohistochemical analysis in ruptured atherosclerotic lesions. MBL predominantly localized around necrotic debris. No MBL was detected in stable lesions (insert). Magnification ×100.

Table 2. Local MBL Gene Expression in Human Liver and Stable (n=8) or Ruptured (n=9) Atherosclerotic Plaques (40 Cycles)

	Mbl2 Gene Expression (C_t)
Human liver	27.3
Stable lesions	ND*
Ruptured lesions	ND*

*MBL gene expression was not detected in individually assessed lesions.

Discussion

The importance of complement in the development of atherosclerosis was first shown around the time that MBL was discovered in 1979[25] and has been corroborated since then. In 1998, 19 years after the detection of complement in atherosclerosis, a role for the lectin pathway was first proposed in atherogenesis.[7] Since then, various contrasting reports suggested an intricate role for MBL in atherosclerosis. The apparently complex role of MBL during atherogenesis underscores the necessity for further study of the thus far poorly understood characteristics of MBL in atherosclerosis.[26]

Our results describe for the first time the deposition and distribution characteristics of MBL in early and advanced atherosclerotic lesions. More importantly, the data clearly suggest that the ability of myeloid cells to express MBL during atherogenesis influences atherosclerosis development.

The knowledge that the variation in plasma MBL levels in humans is regionally distributed around the world, with ≈5% to 10% lacking functional MBL, has led to many studies that investigated MBL as a modifier of disease.[6,27] Individuals that carry variant MBL alleles have an increased risk of infection and acute respiratory tract infections during early childhood, systemic lupus erythematosus, and meningococcal disease.[28–31] In selected patient populations, well-characterized MBL gene variants have also been associated with an increased risk of thrombosis in systemic lupus erythematosus patients and heterosis in relation to mortality in patients in intensive care.[32,33] Population-based studies addressing the role of MBL in atherosclerosis analyzed either MBL genotypes or resulting MBL serum levels in numerous different patient cohorts. From this work, it has been suggested that functional MBL might control atherosclerosis by ensuring the rapid clearance infectious agents, such as *C pneumoniae*, associated with atherosclerosis development.[7,34] Moreover, there is an extensive search for the evolutionary advantage of low functional MBL plasma levels.[35,36]

To understand a possible role of MBL in atherogenesis, different known functions of MBL must be considered. Besides an effective regulator of complement activation, functional MBL can act as a potent opsonin of exogenous (infectious agents) or endogenous danger signals, such as late apoptotic and necrotic cells and cellular debris, which are associated with atherosclerosis development and plaque stability.[37] Consequently, our results might indicate a differential role of MBL in atherogenesis. On the one hand, MBL expression during early atherogenesis may facilitate the rapid clearance of endogenous danger signals in the early atherogenic process, possibly constraining atherosclerosis development.[38,39] On the other hand, increased levels of MBL expression in more advanced atherosclerotic lesions may have a proinflammatory role, as described earlier for coronary artery disease, myocardial infarction, and the risk of cardiac death in patients with type 2 diabetes and rheumatoid arthritis.[15,40,41] Reduced levels of MBL expression, as demonstrated in advanced human and murine atherosclerotic lesions, may illustrate a futile effort to control complement activation, inflammation, and atherosclerosis progression. Interestingly, in its opsonizing capacity, MBL largely resembles the complement component C1q. Recently, C1q was found to reduce atherosclerosis development in atherosclerosis-prone $LDLR^{-/-}$ mice in comparison with C1q/$LDLR^{-/-}$ experimental controls. These findings were largely attributed to the ability of C1q to opsonize apoptotic cells and to facilitate phagocytic clearance in atherosclerotic lesions, a quality similarly observed for MBL.[38,39] Moreover, the C1 complement complex has been shown to bind modified LDL, a major cause of inflammation in arterial vascular disease.[42] Whether these observations also account for the contribution of MBL to atherosclerosis development will be a matter of future research.

Several other complement components have been associated with atherosclerosis development. Reduced complement activity, as seen in C3-, C5-, or C6-deficient mice or rabbit animal models, clearly affects atherosclerosis development.[3,43–46] Atherosclerosis development in C3-deficient $LDLR^{-/-}$ as well as $LDLR^{-/-}$/apolipoprotein $E^{-/-}$ mice was shown to be dependent on an altered lesion progression and lipid metabolism, resulting in increased lesion size compared with normal $LDLR^{-/-}$ and $LDLR^{-/-}$/apolipoprotein $E^{-/-}$ control mice.[43,44]

Our findings suggest an important contribution of extrahepatic, myeloid-derived MBL gene expression to atherosclerosis development. MBL gene expression by myeloid-derived cells has been reported before.[24] Moreover, the importance of extrahepatic MBL expression was clearly demonstrated from work on allogenic hemopoietic stem cell transplantations. Recipients of bone marrow from MBL variant donors had a >4-fold increased risk of serious infection compared with recipients of bone marrow derived from individuals without coding mutations in the *mbl2* gene.[47] The importance of extrahepatic complement, including MBL expression, in various pathologies led us to investigate the role of macrophage-specific MBL expression in atherosclerosis development. However, a systemic role of MBL in atherosclerosis should be further addressed by generation of MBL $A/C^{-/-/-/-}$ triple knockout animals. Such data could provide further insight into the role of circulating MBL in the epidemiological findings of MBL in vascular disease.[14,15,40,41]

Preliminary analysis of advanced human atherosclerotic lesions and MBL gene expression and MBL deposition supports our experimental murine data. Similar to observations in experimental atherosclerotic lesions, no MBL gene expression was observed in advanced human atherosclerotic plaques, suggesting a plasma origin of MBL during advanced stages of atherosclerosis development in mice and humans.

In conclusion, our data clearly demonstrate the deposition and local gene expression of MBL over the course of atherosclerosis development. Deposition and gene expression

of MBL-A and -C were putatively attributed to a protective role for intimal macrophages in murine atherogenesis. The observation that MBL gene expression by myeloid cells controls lesion development is of major importance in unraveling cause and effect mechanisms between MBL and atherosclerosis development. Further studies on the role of versatile immunoregulatory proteins as complement proteins in atherosclerosis will undoubtedly generate new insights that increase our understanding of atherosclerosis as well as augment future treatment possibilities.

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Disclosures

Dr Buurman is a shareholder of the company Hbt that provided some of the monoclonal antibodies. These monoclonal antibodies, as used in this article, are commercially available worldwide. The authors have no further financial conflict of interest.

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CLINICAL PERSPECTIVE

Since an initial publication in 1998, population-based studies identified mannose-binding lectin (MBL) as a modifier of atherosclerosis development; both proatherogenic and antiatherogenic roles of MBL were demonstrated. However, as stated by G.K. Hansson in his 2006 *Arteriosclerosis, Thrombosis, and Vascular Biology* editorial, "confusion prevails." The mechanisms by which MBL influences atherosclerosis development are unknown, and epidemiological data are conflicting, emphasizing the need for additional experimental studies. MBL is considered to be an important initiating complement component with immune regulatory properties and considerable variation in plasma levels between individuals. Its function ranges from complement activation to the MBL-mediated uptake of late apoptotic cells, cellular debris, and foreign organisms by macrophages. In the present study, local MBL-A and MBL-C gene expressions were demonstrated in murine atherosclerotic lesions. Interestingly, mice carrying MBL-A and -C double deficient macrophages had increased (30%) atherosclerotic lesions compared with wild-type controls. Furthermore, the MBL-A and -C distribution pattern observed in the present study suggests that MBL may play a differential role in the atherogenic process. Low MBL levels, although possibly disadvantageous during early atherosclerosis development because of a defect in removal, may well be able to reduce inflammation and subsequent atherosclerosis development in advanced stages of atherosclerosis. This hypothesis would support in large part the previous and often conflicting studies on the role of MBL in atherosclerosis development.

Macrophage-Specific Expression of Mannose-Binding Lectin Controls Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice

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Mice and preparation of tissues

In order to limit the use of experimental animals some LDLR-/- animals enrolled in these studies served as controls in experiments as described before.[1] Animals were fed a high-fat Western diet (n=10) without cholate containing 16% fat, 0.15% cholesterol (Arie Blok B.V., Woerden, the Netherlands) diet for 10 (early) or 18 (advanced) weeks. After anaesthesia the hearts and aortic arches were excised and embedded in OCT compound and frozen on dry ice and subsequently stored at -70^{0}C. Cryostat sections (7μm) were cut with a 42 μm interval, subsequently dried and stored. Sections were fixed in -20^{0}C acetone before immunohistochemistry.

Immunohistochemical staining

Primary antibodies were incubated (1-10 μg/ml) overnight at 4^{0}C in TBS containing 2% normal goat serum (NGS) or 0.1% BSA. Secondary peroxidase or biotin conjugated goat anti rat or goat anti rabbit polyclonal antibodies (Jackson ImmunoResearch, West Grove, PA) were incubated in their appropriate dilutions. Following signal enhancement in case of biotin conjugated secondary antibodies (hMBL) using peroxidase conjugated avidin-biotin complexes (Dako, Glostrup, Denmark), antibody binding was visualized using 3-amino-9-ethylcarbazole (AEC) with H_2O_2 as substrate. Antibody specificity was determined by western blotting under reduced conditions (hMBL, specific band at ±31kD), inhibiting primary antibody binding by specific protein blockage (hMBL), incubating slides with isotype control rat IgG (Macrophage, MBL-A, MBL-C), control rabbit serum (hMBL) or secondary antibody only.

Reverse Transcription-Polymerase Chain reaction

PCR reactions (40 cycle standard) with specific primers were performed using 2μl standard dilutions of cDNA (5ng/μl). The sequences of oligonucleotide primers were: MBL-A, 5'-CCA AAG GGG AGA AGG GAG AAC-3' (forward) and 5'-GCC TCG TCC GTG ATG CCT AG-3' (reversed); MBL-C, 5'-GAC GTG ACG GTG CCA AGG G-3' (forward) and 5'-CTT TCT GGA TGG CCG AGT TTT C-3' (reversed).[2]

Blood and chimerism analysis

Cells were stained either with Mac1-PE and Gr1-FiTC or with 6B2-PE and KT3-FiTC (BD Sciences, San Diego, CA) in PBS 5% normal mouse serum and 1% FCS. After 1 hour, cells were washed and analyzed by FACS analysis (Facssort, BD Sciences) according to following principles: T-cells KT3 positive, B-cells 6B2, monocytes Mac1 positive/GR1 negative, and granulocytes Mac1/GR1 positive. Chimerism was determined as described before.[3]

Atherosclerosis assessment and lesion analysis

The animals were dissected, and hearts and aortic arches were removed and bisected perpendicularly to the heart axis, just below the atrial tips. The tissue was frozen in Tissue-Tec (Shandon, Veldhoven, the Netherlands) with the base facing downward. Cryostat sections were prepared as described above. The aortic lesion area was analyzed using serial sections with 42 μm intervals, beginning from the onset of the aortic valves until the valves had disappeared. The collected sections were stained with toluidine blue and digitally photographed and quantified using Adobe Photoshop (Adobe Systems, San Jose, CA).

Human atherosclerotic lesions

4μm sections were cut from paraffin embedded tissue specimens. Total RNA was isolated as described above. cDNA synthesized from human liver derived total RNA isolate served as positive control sample for hMBL. Specific primers (Sigma) for amplification of hMBL were designed using the Primer Express software package (Applied Biosystems, Foster City, CA) and tested for amplification of contaminating genomic DNA. To minimize the risk of genomic amplification the primers were positioned on different exons. Primer concentrations were optimalized and dilution curves were made from human liver cDNA standard pool to ensure an exponential Taqman amplification for each primer set. The sequences of oligonucleotide primers were: MBL, 5'-GGGTCACCAGGACCAAAGG-3' (forward) and 5'-CAGCCAGGCTACTATCACCAT-3' (reversed).

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