Antiatherosclerotic Effect of an Antibody That Binds to Extracellular Matrix Glycosaminoglycans


Objective—Subendothelial retention of proatherogenic lipoproteins by proteoglycans is critical in atherosclerosis. The aim of this study was to characterize the recognition and antiatherogenic properties of a chimeric monoclonal antibody (mAb) that reacts with sulfated molecules.

Methods and Results—chP3R99 mAb recognized sulfated glycosaminoglycans, mainly chondroitin sulfate (CS), by ELISA. This mAb blocked ≈70% of low-density lipoprotein (LDL)–CS association and ≈80% of LDL oxidation in vitro, and when intravenously injected to Sprague-Dawley rats (n=6, 1 mg/animal), it inhibited LDL (4 mg/kg intraperitoneally, 1 hour later) retention and oxidation in the artery wall. Moreover, subcutaneous immunization of New Zealand White rabbits (n=19) with chP3R99 mAb (100 µg, 3 doses at weekly intervals) prevented Lipofundin-induced atherosclerosis (2 mL/kg, 8 days) with a 22-fold reduction in the intima-media ratio (P<0.01). Histopathologic and ultrastructural studies showed no intimal alterations or slight thickening, with preserved junctions between endothelial cells and scarce collagen fibers and glycosaminoglycans. In addition, immunization with chP3R99 mAb suppressed macrophage infiltration in aorta and preserved redox status. The atheroprotective effect was associated with the induction of anti-CS antibodies in chP3R99-immunized rabbits, capable of blocking CS-LDL binding and LDL oxidation.

Conclusion—These results support the use of anti-sulfated glycosaminoglycan antibody–based immunotherapy as a potential tool to prevent atherosclerosis. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: antibodies ■ atherosclerosis ■ glycosaminoglycan ■ immunotherapy ■ oxidative stress

Atherosclerosis is the underlying pathology of most cardiovascular events, and it is the main cause of morbidity and mortality in developed countries. In this chronic inflammatory disease, the subendothelial retention of apolipoprotein B (apoB)-containing lipoproteins in the artery wall is the critical initiating step according to the “response-to-retention” hypothesis. Proteoglycans (PGs), mainly those made up of chondroitin sulfate (CS) chains, are the most prominent components of the extracellular matrix (ECM) involved in atherogenesis. This process is mediated by electrostatic interactions between glycosaminoglycan (GAG) chains and basic residues present in apoB-containing lipoproteins, thereby increasing susceptibility to oxidation. Oxidized low-density lipoproteins (oxLDLs) are internalized by macrophages through cell surface scavenger receptors, leading to foam cell formation. Macrophage cell surface PGs are also involved in oxLDL binding and contribute to their uptake and catabolism. Furthermore, lesion progression is characterized by CSPG accumulation at atherosclerosis-prone sites, side chain elongation, and increasing in sulfation degree.

Up to now, therapeutic approaches to atherosclerosis have been largely limited to risk factors, targeting mainly hypercholesterolemia and hypertension. However, the maximum efficacy of these strategies in different clinical trials has been 30% to 40%. Different immunotherapeutic approaches have been evaluated in preclinical and clinical studies not only with the purpose of interfering with lipoprotein metabolism but also of modulating specific immune responses that play key roles in the development of the inflammatory process involved in the pathogenesis of the disease. In contrast, therapies based on vessel wall targets, especially those directed to prevent the subendothelial retention of atherogenic lipoproteins, have been much less explored. Endostatin,
a fragment of collagen type XVIII released by proteolysis, was shown to interfere with lipoprotein retention in subendothelial matrix.\(^\text{16}\) In addition, immunization with apoB100-derived peptides containing 1 of the PG binding sites has been considered a promising strategy to block LDL retention in vivo.\(^\text{17}\) Our therapeutic strategy, not previously explored, is the use of antibodies that through their recognition of proatherogenic GAGs interfere with LDL retention and are also capable of inducing autologous antibodies with similar properties in the immunized animals, acting as idiotype vaccines.\(^\text{18}\) We have previously demonstrated the induction of idiotype cascades by an idiotype vaccine in cancer patients.\(^\text{19–21}\)

P3 is a murine monoclonal antibody (mAb) that recognizes several N-glycosylated gangliosides and sulfatides.\(^\text{22}\) P3 mAb has the unusual property of generating a strong anti-idiotype response when administered in a syngenic animal model, even in the absence of adjuvants or carrier proteins.\(^\text{23}\) The chimeric mouse/human IgG1 variant of P3 mAb (chP3) maintained the specificity and idiotype immunogenicity of the murine antibody.\(^\text{24}\) Mutation of arginine residues at heavy chain complementarity determining region (H-CDR1 and 3) showed that they were crucial for P3 mAb antigen binding.\(^\text{25}\)

Recently, researchers obtained a chP3 mutant with an additional arginine in position 99 of H-CDR3 (Kabat numbering; chP3R99), displaying higher reactivity with antigens and more dramatically with sulfatides.\(^\text{26}\)

The purpose of this study was to characterize the reactivity of chP3R99 mAb to GAGs, its effect in foam cell formation, and its capacity to inhibit LDL retention and oxidation in the artery wall. Moreover, we investigated the potential of immunization with chP3R99 mAb to induce anti-GAG antibodies and their impact in the development of atherosclerosis in rabbits.

**Materials and Methods**

chP3R99 mAb reactivity to different GAGs was evaluated by ELISA. The effect of chP3R99 mAb in foam cell formation was studied through its capacity to block oxLDL uptake by J774.A1 macrophage cells. Inhibition of LDL-CS binding by chP3R99 mAb, rabbit sera, and IgG from immunized rabbits was performed by competitive ELISA, and their capacity to prevent LDL oxidation in vitro was analyzed by monitoring malondialdehyde (MDA) formation. The capacity to block LDL retention and oxidation in the artery wall was evaluated in rats. To assess the antiatherogenic effect of chP3R99 mAb in vivo, New Zealand White rabbits were subcutaneously immunized with low doses of the mAb before daily administration of Lipofundin MCT/LCT 20% (referred to as Lipofundin) for 8 days. Aortic arches were used for histopathology, ultrastructural, and redox evaluation. Immunohistochemical studies were performed for detection of macrophages and CD4 and CD8 lymphocytes in rabbit aortas. Serum lipid parameters were measured using Randox Ltd kits, and redox variables were spectrophotometrically determined in supernatants from homogenates of rabbit aortas.

Induction of anti-GAG antibodies by chP3R99 mAb immunization was evaluated in rabbit sera by ELISA. A full description of the methods is given in the online-only Data Supplement.

**Results**

**chP3R99 mAb Reacted With Sulfated GAGs and Human Atherosclerotic Lesions**

It was previously demonstrated that P3 mAb reacts with sulfatides through electrostatic interactions.\(^\text{22}\) Because of the relevance of sulfation degree of arterial GAG chains in atherogenesis,\(^\text{11}\) we evaluated the reactivity of chP3R99 mAb to these molecules. The antibody recognized heparin (Figure 1A), heparan sulfate (Figure 1B), and dermatan sulfate (DS) (Figure 1D) in a similar way, despite differences in sulfation pattern among these GAGs. Interestingly, the highest reactivity was observed for CS (Figure 1C). It also recognized human aortic PGs (Figure 1F) and decorin (not shown), made up of CS and DS GAGs. In contrast, chP3R99 mAb showed a lower reactivity with hyaluronic acid (Figure 1E), a non–sulfate-containing GAG. Besides, chP3R99 mAb recognized GAGs with higher affinity than the parental chP3 mAb, whereas no reactivity was detected for chP3S98 mAb, a mutated variant of P3 where the arginine residue at position 98 of H-CDR3 showed low reactivity with hyaluronic acid (Figure 1E), a non–sulfate-containing GAG. Besides, chP3R99 mAb recognized GAGs with higher affinity than the parental chP3 mAb, whereas no reactivity was detected for chP3S98 mAb, a mutated variant of P3 where the arginine residue at position 98 of H-CDR3 was replaced by serine.\(^\text{25}\)

In addition, chP3R99 showed low or no reactivity with fibronectin, laminin, collagen I, fibrinogen, LDL, oxLDL, 1-palmitoyl-2-arachidonyl-3-phosphorylcholine, or 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (Figure 1 in the online-only Data Supplement).

Furthermore, immunohistochemical studies revealed a strong reactivity of chP3R99 mAb to human atherosclerotic lesions from the aortic arch (Figure IIC–III in the online-only Data Supplement). Also, the antibody recognized foam cells in the intima layer (Figure IIF in the online-only Data Supplement). Similarly, chP3 mAb reacted to human athero-
sclerotic lesions, although reactivity was weaker than that observed for chP3R99 mAb (Figure IIB–IIH in the online-only Data Supplement). No reactivity was observed for chP3S98 mAb (Figure IIA–IIG in the online-only Data Supplement).

chP3R99 mAb Did Not Inhibit oxLDL Uptake by J774A.1 Cells
The significant amounts of cell surface PGs on J774A.1 cell membrane, and their involvement in oxLDL uptake and catabolism, prompted us to study the possible effect of chP3R99 mAb in foam cell formation. First, binding to J774A.1 cells was determined by flow cytometry. More than 95% of the cells were recognized by chP3R99, whereas no reactivity was observed with chP3S98 mAb (Figure IIIA in the online-only Data Supplement). Furthermore, the antibody blocked ~80% of the recognition of this cell line by an anti-CS antibody (Figure IIIB in the online-only Data Supplement). However, high concentrations of chP3R99 mAb blocked neither b-oxLDL binding to cells nor their uptake by J774A.1 cells (Figure IIIC and IIID in the online-only Data Supplement).

chP3R99 mAb Inhibited LDL Retention and Oxidation
To evaluate the ability of chP3R99 to inhibit LDL-CS association in vitro, CS-coated immunoassay plates were coincubated with b-LDL and different amounts of the chimeric antibodies. chP3R99 mAb inhibited the binding of b-LDL to CS in a dose-dependent manner, reaching ~70% inhibition with 80 µg/mL of this mAb. At this concentration, chP3 mAb inhibited only 36%. In contrast, chP3S98 had no effect, whereas the maximum inhibition was obtained when unlabeled LDL was used as competitor (Figure 2A).

To evaluate whether the capacity to block binding of LDL to CS would have further implications in LDL oxidation, we performed an LDL oxidation inhibition assay in vitro. In the presence of CS, LDL was ~2-fold more susceptible to undergoing Cu²⁺ oxidation. chP3R99 mAb inhibited ~75% of MDA formation, whereas no inhibition was detected for hR3 mAb, used as isotype-matched control (Figure 2B).

Further exploration of the chP3R99 mAb effect on LDL retention and oxidation in the artery wall was done in rats. By means of CBLDL3 and EO6 mAbs, LDL and oxLDL were detected in the arterial endothelium, intima, and media 24 hours after intraperitoneal injection of LDL in rats that received phosphate-buffered saline (PBS) or the isotype-matched control hR3 mAb (Figure 2C–2E). In contrast, intravenous administration of chP3R99 mAb 1 hour before LDL resulted in a clear reduction in arterial retention and oxidation of these particles. Accordingly, chP3R99 mAb was detected in rat artery wall, suggesting an inhibitory effect on LDL retention and subsequent oxidative modifications (Figure 2C).

chP3R99 mAb Prevented Atherosclerosis in New Zealand White Rabbits
To assess the antiatherogenic effect of chP3R99 mAb in vivo, New Zealand White rabbits were subcutaneously injected with low doses of the mAb before Lipofundin infusion. Macroscopic and microscopic examination of organs did not show any relevant disease or abnormalities. The body weight did not vary significantly in any experimental group on Lipofundin administration. Microscopic analysis of aortic arch sections from control rabbits showed neither intimal thickening nor distortion in the vascular architecture (data not shown). Meanwhile, intravenous infusion of Lipofundin induced aortic atherosclerotic lesions in all PBS-receiving rabbits and those previously injected with hR3 (Figure 3) or chP3 (data not shown) mAbs. Lesions were characterized by intimal thickening with accumulation of abundant subendo-

Figure 2. Effect of chP3R99 monoclonal antibody (mAb) in low-density lipoprotein (LDL) retention and oxidation. A, Inhibition of LDL binding to chondroitin sulfate (CS) in vitro. ELISA plates were coated with 10 µg/mL CS. Biotin-conjugated LDL (2.5 µg/mL) was coincubated with different concentrations of the chimeric mAbs. B, Inhibition of LDL oxidation in vitro. LDL oxidation was measured as nmol/L malondialdehyde (MDA) produced by preincubating CS (100 µg/mL) with mAbs (1 mg/mL) followed by the addition of LDL (25 µg/mL) and CuSO₄ (10 µmol/L). Percentages of inhibition were calculated relative to the maximum of LDL oxidation dependent on the binding to CS. C, Immunohistochemical staining of aortic sections from rats treated with 1 mg of chimeric antibodies before intraperitoneal injection of LDL (4 mg/kg). LDL and oxidized LDL (oxLDL) were detected with CBLDL3 and EO6 mAbs (10 µg/mL), respectively. Accumulation of chP3R99 mAb in the artery wall was detected with a peroxidase-conjugated goat anti-human IgG antibody (magnification ×20). Scale bars = 100 µm. PBS indicates phosphate-buffered saline. D and E show the percentages of CBLDL3⁻ and EO6⁻ areas with respect to total area. Data are mean±SEM.
thelial extracellular material. Alcian Blue staining revealed increased quantities of GAGs in the artery wall (Figure 3A and 3D). We also observed media-to-intima migration of smooth muscle cells (SMCs) and abundant collagen fibers (Figure 3B and 3E). Conversely, the immunization with chP3R99 mAb prevented atheromatous lesions in 8 rabbits, whereas minor lesions were observed in the 6 remaining animals. In the latter, only slight thickening of the intima was observed with scarce quantities of GAGs and collagen fibers. SMCs showed spindle-like morphology and were longitudinally arranged (Figure 3G and 3H). The immunization with chP3R99 mAb prevented atheromatous lesions in 8 rabbits, whereas minor lesions were observed in the 6 remaining animals. In the latter, only slight thickening of the intima was observed with scarce quantities of GAGs and collagen fibers. SMCs showed spindle-like morphology and were longitudinally arranged (Figure 3G and 3H). The immunization with chP3R99 antibody variant with impaired Fc receptor (FcγR) and complement binding (chP3R99-LALA) also prevented Lipofundin-induced atherosclerosis in 4 rabbits, and only minor lesions were detected in 1 animal (Figure IV in the online-only Data Supplement).

Immunohistochemical studies performed in fresh frozen sections of aortas showed a strong RAM-11 staining in the artery wall (Figure 3A and 3D). We also observed media-to-intima migration of smooth muscle cells (SMCs) and abundant collagen fibers. SMCs showed spindle-like morphology and were longitudinally arranged (Figure 3G and 3H). The immunization with chP3R99 antibody variant with impaired FcγR and complement binding (chP3R99-LALA) also prevented Lipofundin-induced atherosclerosis in 4 rabbits, and only minor lesions were detected in 1 animal (Figure IV in the online-only Data Supplement).

Morphometric analyses of aortic arches showed that Lipofundin produced a significant increase in intima-to-media ratio (1.750 ± 0.340) compared with control rabbits (0.015 ± 0.004), P < 0.001. Immunization with chP3R99 led to a 22-fold reduction in intima-to-media ratio (0.08 ± 0.05) compared with Lipofundin group (P < 0.001).

Further transmission electron microscopy ultrastructural examination confirmed the results observed by light microscopy. No alterations in the aortic artery wall of control rabbits were observed, characterized by a thin intima layer with SMCs immediately underlying the endothelium (Figure 4A–4C). In contrast, Lipofundin caused striking alterations in the endothelium of animals that received PBS, with loss of intercellular junctions (Figure 4D). Additionally, the subendothelial space was thickened, with accumulation of basement membrane-like material, high amounts of collagen fibers (in both transverse and longitudinal dispositions), and

Figure 3. Effect of chP3R99 monoclonal antibody (mAb) on Lipofundin-induced atherosclerosis in New Zealand White (NZW) rabbits. The mAbs were subcutaneously injected (100 μg in phosphate-buffered saline [PBS], 3 doses at weekly intervals) before atherosclerosis induction by intravenous administration of 20% Lipofundin (2 mL/kg) for 8 days or PBS in control groups. Alcian blue, Masson trichromic, and RAM-11 stainings (magnification ×20) showed intimal thickening with distortion in smooth muscle cell arrangement and accumulation of glycosaminoglycans (GAGs) (A) and collagen (B), and macrophage infiltration (C) in aortas from Lipofundin-treated rabbits and those receiving hR3 isotype-matched control (D–F). Only minor lesions were observed in chP3R99-treated rabbits with scarce quantities of GAGs (G) and collagen fibers (H) and no macrophage infiltration (I). Scale bars = 50 μm.
abundant extracellular lipid vacuoles (Figure 4E). We also detected high number of foam cells derived both from macrophages and SMCs (Figure 4F). Similar characteristics were observed in hR3-receiving rabbits, with SMC dedifferentiation to ECM-producing myofibroblasts (Figure 4G-I), and chP3-treated rabbits (data not shown). In contrast, no endothelial damage was detected in any of the chP3R99-receiving animals (Figure 4J). Subendothelial space from rabbits that developed atherosclerotic lesions in this group was characterized by ECM accumulation with scarce collagen fibers (Figure 4K and 4L).

Histopathologic and ultrastructural results together showed that Lipofundin-treated rabbits manifested human-like type III or IV atherosclerotic lesions, chP3- or hR3-receiving animals (Figure 4J). Subendothelial space from rabbits that developed atherosclerotic lesions in this group was characterized by ECM accumulation with scarce collagen fibers (Figure 4K and 4L).
animals were classified as type III lesions, and chP3R99-immunized animals showed no lesions (4 of 9) or type I atherosclerotic lesions (5 of 9).

### Table. Lipid Profile and Redox Biomarkers

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<th>Control</th>
<th>Lipofundin</th>
<th>chP3R99</th>
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<th>hR3</th>
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<td>TC, mmol/L</td>
<td>1.71±0.04*</td>
<td>2.96±0.10†</td>
<td>2.92±0.05†</td>
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<td>TG, mmol/L</td>
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<td>2.77±0.05†</td>
<td>2.37±0.10†</td>
<td>2.60±0.05†</td>
<td>2.24±0.09†</td>
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<td>HDL-C, mmol/L</td>
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<td>1.12±0.03†</td>
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<td>LDL-C, mmol/L</td>
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<td>0.80±0.03‡</td>
<td>0.87±0.03‡</td>
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<td>Aortic redox biomarkers</td>
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<td>n=8</td>
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<td>n=5</td>
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<td>PP, μmol/mL of MDA/mgPr</td>
<td>13.59±0.91*</td>
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<td>MDA, (μmol/mL/mgPr)</td>
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<td>28.13±1.12†</td>
<td>18.43±1.12*</td>
<td>18.66±0.94*</td>
<td>25.18±2.19†</td>
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<td>AOPP, (μmol/mL of chloramines/mgPr)</td>
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<td>15.98±1.21‡</td>
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<td>SOD, (U · mL⁻¹ · min⁻¹)/mgPr</td>
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<td>73.93±2.94†</td>
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<td>89.34±5.81§</td>
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Values are mean±SEM. TC indicates total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PP, peroxidation potential; MDA, malondialdehyde; mgPr, mg of protein; AOPP, advanced oxidation protein product; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione.

*†‡§Values with different symbols are significantly different (P<0.05).

The Antiatherogenic Effect Induced by chP3R99 Immunization Was Mediated by the Generation of Anti-CS Antibodies Capable of Inhibiting LDL Binding to CS and Their Oxidation

Induction of anti-GAG antibodies in rabbit sera was tested by ELISA. An antibody response against CS and DS was detected only in chP3R99-immunized animals (Figure 5A; Figure VA in the online-only Data Supplement), although lower for the latter (ratios of post/pre of 6.1 and 2.38, respectively). Increases in the reactivities with heparin (ratio of post/pre, 2.87), heparan sulfate (ratio of post/pre, 2.24), and hyaluronic acid (ratio of post/pre, 3.6) were observed in sera from chP3R99- and chP3-immunized animals (Figure VB–VD in the online-only Data Supplement). IgG antibodies purified from chP3R99-immunized rabbits showed a similar pattern of anti-GAG reactivity to sera from this group (Figure 5A; Figure V in the online-only Data Supplement). These results suggested that although both chP3R99 and chP3 mAbs were able to induce antibody responses against GAGs, the antibody response against CS and DS induced by chP3R99 was the only one associated with its antiatherogenic effect of chP3R99 immunization.

No reactivity was detected against ECM proteins, LDL, or oxLDL either in the sera (data not shown) or in the IgG fraction purified from nonimmunized or chP3R99-immunized rabbit sera (Figure VI in the online-only Data Supplement). No human IgG was detected in chP3R99-immunized rabbit sera or in the purified IgG fraction at the time of euthanization (data not shown).

We next evaluated the capacity of chP3R99-immunized rabbit sera and purified IgG to inhibit the binding of b-LDL to CS. As shown in Figure 5B, sera from chP3R99-
immunized rabbits (1:50) and their IgG antibody fraction (25 μg/mL) were able to block LDL-CS association in 60% and 39.4%, respectively. In contrast, no inhibitory effect was observed with sera from hR3-immunized rabbits. chP3R99, a P3 mAb mutant in which glutamic acid 99 (Kabat numbering) was replaced by arginine, increased the density of positive charge on H-CDR3 and consequently the affinity to their glycolipidic ligands,25 also had a higher reactivity against GAGs than the parental chP3 antibody. On the contrary, chP3 mutants with lower arginine content as chP3S98 mAb25 totally lost the reactivity with these antigens. Similar behavior was observed for the reactivity with human atherosclerotic plaques.

It was previously demonstrated that electrostatic interactions govern the specificity of P3 mAb, either with the carboxyl group of sialic acid or sulfated groups in sulfatides.22,25 In fact, the lowest reactivity of chP3R99 was with hyaluronic acid, indicating that the lack of sulfate groups affected the antibody reactivity. On the other hand, because of the high sulfate content of heparin, we expected the highest reactivity with this GAG, but in contrast, the antibody preferably reacted with CS. This observation could be explained because, unlike heparin and heparan sulfate, CS contains sulfated galactosamine residues in the disaccharide units, similar to the epitope recognized by P3 mAb in sulfatides.22 Also, the antibody recognized human aortic PGs and decorin, which contains CS and DS as GAG side chain.

The role of arterial PGs has long been demonstrated in atherosclerosis.5,9,27 On interaction with intimal CSPGs, LDL particles undergo structural and functional modifications, thereby increasing their atherogenicity.2,28 In particular, oxLDL induces endothelium and SMCs to express monocyte chemotactic activity and directly acts as a chemoattractant to monocytes, SMCs, and T lymphocytes.2 Also, macrophage cell surface PGs are involved in the binding, uptake and catabolism of oxLDL, acting both as coreceptors for scavenger receptors (SR-A and CD36) and as a direct gate for oxLDL phagocytosis.10 Although chP3R99 mAb recognized murine macrophages and inhibited the recognition of an anti-CS antibody to these cells, it did not have a direct impact...
on either the oxLDL binding to plasma membrane or foam cell formation. These results are not surprising because a high number of receptors have been shown to mediate the binding and uptake of oxLDL to macrophages. In fact, interference in PGs expression either with NaClO3, B-D xylose, or glycosidases has led only to a partial decrease in oxLDL binding.8,10

Then, we assessed the inhibitory ability of chP3R99 mAb in the binding of LDL to CS as a potential mechanism to avoid the retention of LDL in the arterial intima. Our results showed that chP3R99 blocked $\approx 70\%$ the binding of LDL to CS and $\approx 80\%$ of their oxidation in vitro. Moreover, intravenously administered chP3R99 mAb accumulated in the artery wall of rats, thereby inhibiting subendothelial LDL entrapment. Consequently, there was a marked reduction of LD oxidation in the artery wall of rats 24 hours after LDL injection, which, as demonstrated previously,29 can be detected 6 hours after LDL administration. Hence, it is likely that this blocking property is exerted through the binding of this mAb to arterial GAGs, which could result in an antiatherogenic effect.

In fact, administration of chP3R99 mAb in low doses prevented atherosclerosis in New Zealand White rabbits. It was previously reported30,31 that daily infusion of Lipofundin for 8 days induces aortic lesions characterized by intima thickening and increased presence of collagen fibers. In addition, we detected the presence of large amounts of foam cells, of both macrophage and SMC origins, as well as SMC migration and transformation from contractile to synthetic phenotype, with production of abundant collagen fibers and GAGs. These characteristics, along with the presence of abundant extracellular lipid vacuoles, fibrillogenesis, and endothelial injury, demonstrated the induction of advanced lesions, resembling human type III to IV lesions. In contrast, chP3R99 mAb reduced 22-fold the intima-to-media ratio in rabbits. In these animals, either no lesions at all were observed, or only slight intimal thickening characterized by very few GAGs and collagen, resembling human type I lesions.32

Also, the administration of 20% Lipofundin produced a significant increase in serum lipid variables in all Lipofundin-receiving groups. There is previous evidence that infusion of 10% Lipofundin fat emulsions produces an acute increase of $\approx 60\%$ in serum total cholesterol in humans and can cause lipids and apolipoproteins to be exchanged with lipoproteins.33 Hence, the antiatherogenic effect of chP3R99 mAb was exerted by mechanisms other than serum lipid modulation.

As mentioned, an essential step in the onset and progression of atherosclerosis is the oxidation of LDL particles, which is favored on LDL entrapment in the artery wall39 and yields a wide range of biologically active products.40 In our study, Lipofundin-induced lesions were accompanied by disruption in prooxidant/antioxidant balance and a decrease in NO$^-$ bioavailability, as previously observed by our group.35 Immunization with chP3R99 mAb protected the redox environment in aorta, which was consistent with the capacity of this mAb to inhibit LDL oxidation in vitro. Although most of oxidation biomarkers were affected by Lipofundin, these variables were lower in chP3R99-immunized rabbits than in animals that were treated with isotype-matched control mAbs or that received PBS before Lipofundin administration. For instance, AOPP levels were lower in this group, whereas peroxidation potential and MDA concentrations remained unaltered. High plasma concentration of MDA has long been used as a marker of oxidative stress and progression of atherosclerosis36 and is closely associated with plaque instability and thrombotic events.37,38 Meanwhile, AOPPs are preferentially formed by chlorinated oxidants produced by myeloperoxidase and mediate inflammation and oxidative signaling.39

Most of the early oxidative modifications undergone by vascular ECM-retained atherogenic lipoproteins are triggered by free radicals, especially O$_2^-$ anion.40 We found an increased superoxide dismutase activity on Lipofundin administration, suggesting a possible O$_2^-$ overproduction, and consequently of catalase, to eliminate the H$_2$O$_2$ generated. In contrast, the activity of these enzymes was significantly lower in rabbits treated with chP3R99 mAb than in groups that received control mAb or PBS before the administration of Lipofundin. It has been reported that there is an increase in vascular NADPH oxidase activity, the main source of O$_2^-$ in the artery,40 and also of catalase in response to H$_2$O$_2$ and lipoperoxides produced during atherogenesis.41 It should be noted that in most of atherosclerosis animal models induced by high-fat/high-cholesterol diets, the disease develops for several months, with the establishment of chronic inflammation.42 However, our model is characterized by the acute induction of atherosclerosis by intravenous infusion of lipid emulsion. Thus, it is possible that the increased activity of these enzymes observed in our model could be related to homeostatic mechanisms, whereas long-term atherosclerosis models could promote a decrease in the activity of these enzymes.43

Meanwhile, the occurrence of oxidative stress is closely related to deprivation of antioxidant defenses, such as GSH, a potent free radical scavenger. GSH is involved in the maintenance of intracellular redox environment and the regeneration of enzymes such as GSH peroxidases.44 Administration of chP3R99 mAb preserved the levels of GSH, in line with the results discussed above. Also, the antibody protected NO$^-$ levels as indicative of endothelial function preservation. In rabbit vascular lesions, the reduced bioavailability of NO$^-$ has been attributed to inactivation of NO$^-$ rather than impairment of its synthesis.45 NO$^-$ is an important regulator for vascular homeostasis that modulates vascular tone and inhibits SMC proliferation, platelet aggregation, and lipid peroxidation.46 These results are consistent with those observed by transmission electron microscopy, where no endothelial damage was found in rabbits immunized with chP3R99 mAb. Modulation of oxidative stress by chP3R99 mAb is very relevant, because a number of oxidized molecules are involved in endothelial dysfunction, leukocyte recruitment and activation, and inflammation establishment.40

In addition, immunohistochemical and ultrastructural studies showed a suppression of macrophage infiltration and foam cell formation in the aortas of rabbits treated with chP3R99. This effect has also been claimed to explain the antiatherogenic properties of human polyclonal immunoglobulins (in-
travenous immunoglobulins) in apoE knockout mice. Interestingly, this effect was shown to be dependent on the Fc portion of intravenous immunoglobulins, as the F(ab')2 failed to ameliorate atherosclerotic lesions and to suppress macrophage accumulation in fatty streak lesions. However, the possible direct antiatherogenic activity and the abrogation of macrophage infiltration induced by chP3R99 were demonstrated to be Fc independent, because in rabbits administered a chP3R99 variant with impaired FcγR and complement binding (chP3R99-LALA), these effects were also verified. Moreover, they were exclusive of chP3R99, as macrophages infiltrated lesions of animals treated with other isotype-matched mAbs, including parental chP3, which has a lower reactivity than chP3R99 for GAGs.

The finding that chP3R99 immunization was able to induce anti-CS antibodies could be explained through the generation of an anti-idiotypic cascade, in which a fraction of the induced anti-idiotypic antibodies (Ab2) behaves as a functional mimic of GAG antigenic determinants, resulting in the generation of a chP3R99 (Ab1)-like anti-anti-idiotypic (Ab3) antibody response. Taking into account all the present results, we can propose that the mechanism(s) involved in the antiatherogenic effect produced by chP3R99 administration in rabbits could be, first, a direct inhibition of the binding of LDL to PGs of the arterial intima by chP3R99 mAb. However, we administered very low quantities of the mAb, and human IgG was not detected in either the serum or the aorta of immunized rabbits. A second and more probable mechanism could be mediated by the anti-CS antibodies generated by immunization with chP3R99. In fact, we demonstrated not only that the anti-CS antibody response was associated with the antiatherogenic effect of chP3R99 immunization but also that the serum antibodies were capable of inhibiting LDL-CS association and their oxidation in vitro. Thus, the inhibition of subendothelial retention and oxidation of proatherogenic lipoproteins by anti-CS antibodies can prevent the oxidative stress and the macrophage inflammatory response produced by Lipofundin administration, avoiding or reducing atherosclerotic lesion development. Although in our study, no evident toxicity was observed in the animals treated with chP3R99 mAb, additional toxicological studies are needed to address the potential risks associated with the generation of antibodies against self-antigens such as PGs, which are involved in many physiological processes.

In summary, our results demonstrated that immunization with chP3R99 mAb prevented the development of atherosclerosis lesions, and this effect was associated with its capacity to induce antibodies capable of blocking the binding of LDL to CS and their oxidation, thus acting as an idiotypic vaccine. In addition, our study provides data strongly supporting the response-to-retention hypothesis as the key event in the initiation of atherosclerosis.

Acknowledgments
The authors thank Dalia R. Álvarez and Milagros Frómeta for technical assistance.

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Disclosures
None.

References


Antiatherosclerotic Effect of an Antibody That Binds to Extracellular Matrix Glycosaminoglycans

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Supplement Material

Anti-atherosclerotic effect of an antibody that binds to extracellular matrix glycosaminoglycans.

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Running title: Anti-glycosaminoglycans anti-atherogenic antibody

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**Supplemental Materials and Methods**

**Materials**

Heparan sulfate (HS, bovine kidney), dermatan sulfate (DS), heparin (porcine intestinal mucosa), CS (bovine cartilage) and decorin were purchased from Sigma. Hyaluronic acid (HA, human umbilical cord) was purchased from Fluka-BioChemica. PGs from human aortas (HAPGs) obtained at autopsy, were isolated as described previously\(^1\) and the amounts of PGs were expressed relative to protein content.

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chP3\(^2\), chP3R99\(^3\), chP3S98\(^4\) and humanized R3 (anti-human epidermal growth factor receptor) (hR3)\(^5\) mAbs were purified and conjugated to biotin as described previously.\(^6\) A chimeric R99 antibody variant with impaired Fc gamma receptor (Fc\(\gamma\)R) and complement binding\(^7\) (chP3R99-LALA) was obtained by cloning the chP3R99 heavy chain variable region (VH) gene segment into the pAH4604 (Ala/Ala) vector, which bears two mutations at the human \(\gamma\)1 CH2 region consisting in the replacement of the 234 and 235 leucine residues by alanines. The reduced ability of the purified chP3R99-LALA antibody to interact with Fc\(\gamma\)Rs was proven with the Fc\(\gamma\)RI and Fc\(\gamma\)RII-expressing human monocyte-derived U937 cell line, as described elsewhere.\(^8\)

IgG from rabbit sera were purified using Prosep®-vA Ultra Plus affinity chromatography (Millipore), according to manufacturer instructions.

EO6 mAb, which recognizes oxidized phospholipids, was kindly provided by Dr. C. J. Binder (Medical University of Vienna, Austria); CBLDL3, an anti-apoB-100 mAb, was obtained from Heber Biotech; RAM-11, an anti-rabbit macrophage mAb was purchased from Dako. Anti-human fibronectin, anti-rabbit CD4 (KEN-4) and anti-rabbit CD8 (12.C7) mAbs, were obtained from Serotec. Lipofundin MCT/LCT 20% (referred to as Lipofundin) was purchased from Braun Melsungen.

**Lipoproteins**

LDL were isolated from human plasma by sequential ultracentrifugation and protein content was determined by Lowry assay.\(^9\) LDL oxidization was performed at a final concentration of 200 \(\mu\)g protein/mL in 20 \(\mu\)mol/L CuSO\(_4\).
solution at 37°C for 18h. The oxidation was verified by thiobarbituric acid reactive substance assay and ELISA using EO6 mAb. Native LDL or oxLDL conjugation to biotin was performed as described elsewhere.6

Cells
Murine macrophage cell line J774A.1 (ATCC) was cultured in Dulbecco’s modified Eagles medium (Gibco) supplemented with 10% heat inactivated fetal calf serum 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine (Gibco), and maintained at 37°C, 5% CO₂.

Animals
Sprague-Dawley male rats (250-300 g, 6 weeks old) and New Zealand white (NZW) male rabbits (2.5-3.5 kg, 12 weeks old) were obtained from CENPALAB. Animals were housed under standard conditions (25°C, 60±10% humidity), 12h day/night cycles with water and food ad libitum. All procedures were approved by the Institutional Animal Ethic Committee, in accordance with its animal care and use guidelines.

ELISA
Reactivity of chimeric mAbs, rabbit sera and rabbit IgG to GAGs and HAPGs was evaluated as described previously.10 Briefly, Maxisorp (Nunc) plates were coated with 10 µg/mL of GAGs and HAPGs in HEPES-buffered saline and incubated overnight at 4°C. The samples were added to the plates and incubated for 1h at room temperature (RT). Binding to LDL, oxLDL, laminin, fibronectin, fibrinogen and collagen I was evaluated using an ELISA technique described previously11 in Maxisorp plates coated with the different proteins (10 µg/mL in carbonate buffer, pH 9.6). The reactivity with POVPC and PAPC was detected by ELISA as previously reported.12 PolySorp Immunoplates (Nunc) were coated with 50 µL/well of POVPC or PAPC at 50 µg/mL in 100% ethanol. As secondary antibodies were used alkaline phosphatase (AP)-conjugated goat anti-human IgG (Jackson) or biotin-conjugated goat anti-rabbit IgG antibody followed by AP-conjugated streptavidin complex (Jackson). Absorbance was measured at 405 nm in an ELISA reader. We considered antibody response induction after immunization with a particular chimeric antibody when ratio of postimmune to the preimmune serum absorbance values (post/pre) were ≥ 2.0. Assays were performed in triplicate for each sample and coefficient of variation was <15%. Background values were less than 0.1.
For competitive ELISA, immunoplates were coated with CS (10 µg/mL) in HEPES-buffered saline (HBS) and co-incubated with biotinylated (b)-LDL (2.5 µg/mL) and increasing concentrations of LDL, mAbs, rabbit sera or purified serum IgG in HBS containing 2 mmol/L CaCl₂ and 2 mmol/L MgCl₂. Binding of b-LDL to CS was detected with AP-streptavidin complex (Jackson). Percentages of inhibition were calculated relative to the binding of b-LDL to CS in HBS for mAbs, or in the presence of sera or purified IgG from non-immunized rabbits in the same conditions.

Flow Cytometry

J774A.1 cells (0.5x10⁶/tube) were blocked with 20% human AB serum in phosphate-buffered saline (PBS), biotin-conjugated chimeric mAbs (10 µg/mL) were added, and binding was detected with streptavidin-FITC complex (1:400; BD Bioscience). To determine whether recognition was mediated by cell surface CSPGs, cells were co-incubated with chimeric mAbs (100 µg/mL) and an anti-CS mAb (1:100, Sigma). The latter was detected by staining with FITC-conjugated goat anti-murine IgM antibody (1:400; Jackson). The capacity to block the binding of oxLDL to J774A.1 cells was performed by co-incubating the cells with b-oxLDL (20 µg/mL) and chimeric antibodies (100 µg/mL) for 4h at 4°C, followed by streptavidin-FITC complex (1:400). The effect on oxLDL uptake was performed similarly, but cells were incubated with b-oxLDL at 37°C, permeabilized with 0.1% Triton X-100 in PBS for 10 min followed by the streptavidin-FITC complex. The percentage of stained cells was determined in a FACScan instrument (BD Biosciences). A total of 10⁴ cells were analyzed with the WinMDI 2.8444 software on every assay.

Inhibition of LDL oxidation in vitro

The capacity to inhibit LDL oxidation in vitro of chP3R99 mAb and the antibodies induced by the immunization with this chimeric antibody was evaluated by co-incubating CS (100 µg/mL in PBS) with chP3R99 mAb, purified IgG antibodies from chP3R99-immunized rabbits (1 mg/mL) or sera (1:50) for 45 min at 37°C. Later, LDL (25 µg/mL) and CuSO₄ (10 µM) were added to the mixtures and the kinetic of LDL oxidation for 6h was monitored through MDA formation, determined with the LPO-586 kit (Calbiochem). MDA formation dependent on LDL-CS binding was calculated after subtracting the absorbance values obtained in the absence of CS. Percentages of
inhibition were calculated relative to nM of MDA obtained in PBS, sera or purified IgG from non-immunized rabbits. As controls of specificity were used hR3 mAb or hR3-immunized rabbit sera.

**Inhibition of LDL retention in the artery wall of rats**

The capacity to block LDL retention and oxidation in the artery wall of chP3R99 mAb was evaluated in a rat model described previously. 13 Rats (n=6 per group) were intravenously injected via the tail vein with 1 mg of chP3R99 mAb, hR3 mAb or PBS. After 1h, LDL (4 mg/kg) were intraperitoneally administered, and 24h later the rats were euthanized under anesthesia (80 mg/kg ketamine and 10 mg/kg xylazine HCl, IM) by intracoronary injection of KCl. After perfusion fixing for 5 min with 4% formaldehyde, the aortas were removed and placed in 4% formaldehyde for 24h at 4°C before paraffin embedding.

**Atherosclerosis induction**

Atherosclerosis was induced in NZW rabbits by daily administration of Lipofundin (2 mL/kg, 8 days) through marginal ear vein, as previously described. 14-15 Nineteen rabbits were subcutaneously injected with 100 µg of chP3R99 mAb in PBS, nine with chP3 mAb, five with chP3R99-LALA and 15 with hR3 mAb (3 doses at weekly intervals) and after the last immunization Lipofundin was administered. As controls, 20 rabbits received PBS before Lipofundin and 15 were injected with PBS both subcutaneously and intravenously. Serum samples were obtained at the beginning of the study, a week after the last immunization and after Lipofundin administration and stored at -20°C until use. The animals were anesthetized with ketamine hydrochloride (35 mg/kg and 5 mg/kg xylazine HCl, IM) followed by intracoronary injection of KCl. Defatted aortic arches were placed in 0.1 mol/L Tris-HCl buffer, pH 7.6, with 1 mmol/L EDTA and 0.2 mmol/L butylated hydroxytoluene and macerated for biochemical determinations. Homogenized tissue was centrifuged at 4500g for 20 min, 4°C and supernatants were stored at -80°C until use. For histopathological studies, the aortas were fixed in 4% buffered formaldehyde (pH 7.4) for 24h and samples from the aortic arch were paraffin-embedded or fixed for electron microscopy analysis. Aortic arches were used for histopathology and redox evaluation, due to the preferential development of Lipofundin-induced atherosclerotic lesions in this segment. 14
**Immunohistochemistry**

Paraffin-embedded aortic sections from human atherosclerotic plaques, rats and rabbits were cut into 5-µm slices, deparaffinized, rehydrated, and blocked for endogenous peroxidase activity. Human sections were incubated with biotinylated chimeric mAbs (20 µg/mL) for 30 min at RT, followed by addition of peroxidase-streptavidin complex (1:400; Jackson). Detection of LDL and oxLDL in rat aortas was performed by incubating aortic sections with CBLDL3 and EO6 mAbs (20 µg/mL), respectively, overnight at RT. Then, biotin-conjugated goat anti-murine immunoglobulin antibody (1:400; Jackson) followed by peroxidase-streptavidin complex (1:400; Jackson) was added. To evaluate the accumulation of chP3R99 in rat or rabbit aortas a peroxidase-conjugated goat anti-human IgG antibody was used (1:400, Jackson). Finally, sections were incubated with diaminobenzidine substrate solution and counterstained with hematoxylin. For macrophage detection in rabbit aortas, frozen sections (4 µm) were immunostained with RAM-11 mAb and for CD4 and CD8 lymphocytes detection were immunostained with KEN-4 or 12.C7 mAbs, respectively; followed by the addition of biotinylated goat anti-mouse immunoglobulins antibody. Images of immunostained sections captured digitally with a DP20 camera coupled to an optic light microscope Olympus BX51, were quantified using computerized image analysis (Adobe Photoshop CS3 software).

**Histopathology and morphometry**

Aortic paraffin-sections from rabbits were stained with Masson’s Trichromic and Alcian Blue staining to detect collagen and GAGs, respectively. Morphometrical analyses were performed to calculate intima-media ratio (IMR) in Verhoeff-Van Giesson stained sections. The sections were analyzed in an optic microscope Olympus BX51. Vascular lesions were classified according to the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association.16

**Electron microscopy**

For transmission electron microscopy (TEM), samples from rabbit aortic arch were fixed for 1h at 4°C in 3.2% glutaraldehyde, 0.1 mol/L PBS (pH 7.4) and postfixed in 1% OsO4 for 1h. After graded ethanol dehydration, samples were embedded in Spurr low-viscosity epoxy resin for 24h at 37°C. Ultrathin sections were cut into 400-500 Å thick slice with an LKB Ultramicrotome (Nova LKB), counterstained with uranyl acetate and lead citrate, and analyzed in a JEOL JEEM-2000EX.
Lipid parameters

Total serum cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were measured using Randox Ltd. kits.

Redox biomarkers

Redox variables were spectrophotometrically determined in supernatants from homogenates of rabbit aortas and expressed relative to protein content. The superoxide dismutase (SOD) activity was evaluated by using RANSOD Ltd. Kit and catalase (CAT) activity was assessed by following the H₂O₂ decomposition at 240 nm for 1 min. Glutathione (GSH) was measured as described elsewhere. MDA was determined with a LPO-586 kit (Calbiochem). For peroxidation potential (PP) determination, samples were incubated with 2 mmol/L CuSO₄ for 24h at 37°C. PP was calculated by subtracting the MDA levels in sera from those obtained 24h after lipid peroxidation induction. Advanced oxidation protein products (AOPP) were measured as described previously and expressed as μmol/L of chloramine-T equivalents. Nitrites/nitrates levels were measured as a surrogate marker of NO content in the samples by the Griess reaction. Nitrates were reduced to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA) followed by Griess reagent in 0.25% phosphoric acid (Sigma). Samples were incubated at RT for 10 min and absorbance was measured at 540 nm in a microplate reader (SUMA).

Statistical analysis

Values were expressed as mean±SEM. Statistical analysis was performed with SPSS 11.5 software. For multiples comparisons one-way ANOVA was used followed by Tukey post-test. Values of *P*<0.05 were considered statistically significant.

Supplemental References


Supplemental Figure I. chP3R99 mAb reactivity to proteins, lipoproteins and phospholipids. ELISA plates were coated with 10 μg/mL of fibronectin (A), laminin (B), collagen I (C), fibrinogen (D), LDL (E), oxLDL (F) or 50 μg/mL of PAPC (G) or POVPC (H). Plates were incubated with mAbs followed by an AP-conjugated goat anti-rabbit IgG. Anti-human fibronectin and EO6 antibodies were used as control. Data are mean±SEM.
Supplemental Figure II. chP3R99 mAb reactivity to atherosclerotic plaque from human aorta. Formalin-fixed paraffin-embedded human aortic sections were incubated with biotinylated chP3R99 (C through I), chP3 (B through H) or the isotype-matched chP3S98 (A through G) mAbs (20 µg/mL). The reaction was detected using a streptavidin-peroxidase complex. Arrows show foam cells (40X). Bars=100-µm.
**Supplemental Figure III. Effect of chP3R99 mAb on the oxLDL binding to J774A.1 cells and foam cell formation.**

(A) Recognition of murine macrophage cell line J774A.1 by chP3 mutants. Cells were incubated with biotinylated chimeric mAbs (10 µg/mL), stained with streptavidin-FITC complex, and analyzed by flow cytometry.

(B) Inhibitory effect of chP3R99 mAb on the recognition of J774A.1 cells by an anti-CS antibody. Cells were cocultivated with chimeric (100 µg/mL) and anti-CS (1:100) mAbs, and the latter was detected by adding biotin-conjugated goat anti-murine IgM antibody followed by streptavidin-FITC complex.

(C) Inhibition of b-oxLDL binding to J774A.1 cells. Cells were incubated with the chimeric mAbs (100 µg/mL) and b-oxLDL (20 µg/mL) at 4°C for 4h and the latter were detected with the streptavidin-FITC complex.

(D) The inhibition of b-oxLDL uptake was performed similar to (C), but cells were incubated with b-oxLDL at 37°C for 4h, permeabilized with 0.1% Triton X-100 and stained with the streptavidin-FITC complex. Data are expressed as the percentage of stained cells of at least two experiments.
Supplemental Figure IV. Effect of chP3R99-LALA mAb on Lipofundin-induced atherosclerosis in NZW rabbits. Hematoxillin & Eosin and RAM-11 stainings (20X) showed intimal thickening (A) and macrophage infiltration (B) in aortas from Lipofundin-treated rabbits. In rabbits treated with chP3R99-LALA no lesions (C) and no macrophage infiltration in the aorta were observed (D). Bars=100-µm.
Supplemental Figure V. Anti-GAG response in rabbits immunized with chP3R99 mAb. ELISA plates were coated with DS (A), heparin (B), HS (C) or hyaluronic acid (D) (10 μg/mL) and incubated with sera from PBS group, Lipofundin-receiving animals or mAbs-IR (1:3000). Also, reactivity to CS of purified non-IR IgG or chP3R99-IR IgG (25 μg/mL) was evaluated. Bars represent the ratio of absorbance values of postimmune to preimmune sera or chP3R99-IR IgG to non-IR IgG.
Supplemental Figure VI. Reactivity of purified IgG from chP3R99-immunized rabbits to proteins from the ECM and LDL. ELISA plates were coated with fibronectin (A), laminin (B), collagen I (C), LDL (D) or oxLDL (E) (10 µg/mL). Purified IgG from non-immunized rabbits (IR) or chP3R99-IR were added (25µg/mL) followed by an AP-conjugated goat anti-rabbit IgG. Data are mean±SEM.
Supplement Material

Anti-atherosclerotic effect of an antibody that binds to extracellular matrix glycosaminoglycans.

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Sprague-Dawley male rats (250-300 g, 6 weeks old) and New Zealand white (NZW) male rabbits (2.5-3.5 kg, 12 weeks old) were obtained from CENPALAB. Animals were housed under standard conditions (25°C, 60±10% humidity), 12h day/night cycles with water and food ad libitum. All procedures were approved by the Institutional Animal Ethic Committee, in accordance with its animal care and use guidelines.

**ELISA**

Reactivity of chimeric mAbs, rabbit sera and rabbit IgG to GAGs and HAPGs was evaluated as described previously.\textsuperscript{10} Briefly, Maxisorp (Nunc) plates were coated with 10 µg/mL of GAGs and HAPGs in HEPES-buffered saline and incubated overnight at 4°C. The samples were added to the plates and incubated for 1h at room temperature (RT). Binding to LDL, oxLDL, laminin, fibronectin, fibrinogen and collagen I was evaluated using an ELISA technique described previously\textsuperscript{11} in Maxisorp plates coated with the different proteins (10 µg/mL in carbonate buffer, pH 9.6). The reactivity with POVPC and PAPC was detected by ELISA as previously reported.\textsuperscript{12} PolySorp Immunoplates (Nunc) were coated with 50 µL/well of POVPC or PAPC at 50 µg/mL in 100% ethanol. As secondary antibodies were used alkaline phosphatase (AP)-conjugated goat anti-human IgG (Jackson) or biotin-conjugated goat anti-rabbit IgG antibody followed by AP-conjugated streptavidin complex (Jackson). Absorbance was measured at 405 nm in an ELISA reader. We considered antibody response induction after immunization with a particular chimeric antibody when ratio of postimmune to the preimmune serum absorbance values (post/pre) were ≥ 2.0. Assays were performed in triplicate for each sample and coefficient of variation was <15%. Background values were less than 0.1.
For competitive ELISA, immunoplates were coated with CS (10 µg/mL) in HEPES-buffered saline (HBS) and co-incubated with biotinylated (b)-LDL (2.5 µg/mL) and increasing concentrations of LDL, mAbs, rabbit sera or purified serum IgG in HBS containing 2 mmol/L CaCl₂ and 2 mmol/L MgCl₂. Binding of b-LDL to CS was detected with AP-streptavidin complex (Jackson). Percentages of inhibition were calculated relative to the binding of b-LDL to CS in HBS for mAbs, or in the presence of sera or purified IgG from non-immunized rabbits in the same conditions.

Flow Cytometry

J774A.1 cells (0.5x10⁶/tube) were blocked with 20% human AB serum in phosphate-buffered saline (PBS), biotin-conjugated chimeric mAbs (10 µg/mL) were added, and binding was detected with streptavidin-FITC complex (1:400; BD Bioscience). To determine whether recognition was mediated by cell surface CSPGs, cells were co-incubated with chimeric mAbs (100 µg/mL) and an anti-CS mAb (1:100, Sigma). The latter was detected by staining with FITC-conjugated goat anti-murine IgM antibody (1:400; Jackson). The capacity to block the binding of oxLDL to J774A.1 cells was performed by co-incubating the cells with b-oxLDL (20 µg/mL) and chimeric antibodies (100 µg/mL) for 4h at 4°C, followed by streptavidin-FITC complex (1:400). The effect on oxLDL uptake was performed similarly, but cells were incubated with b-oxLDL at 37°C, permeabilized with 0.1% Triton X-100 in PBS for 10 min followed by the streptavidin-FITC complex. The percentage of stained cells was determined in a FACScan instrument (BD Biosciences). A total of 10⁴ cells were analyzed with the WinMDI 2.8444 software on every assay.

Inhibition of LDL oxidation in vitro

The capacity to inhibit LDL oxidation in vitro of chP3R99 mAb and the antibodies induced by the immunization with this chimeric antibody was evaluated by co-incubating CS (100 µg/mL in PBS) with chP3R99 mAb, purified IgG antibodies from chP3R99-immunized rabbits (1 mg/mL) or sera (1:50) for 45 min at 37°C. Later, LDL (25 µg/mL) and CuSO₄ (10 µM) were added to the mixtures and the kinetic of LDL oxidation for 6h was monitored through MDA formation, determined with the LPO-586 kit (Calbiochem). MDA formation dependent on LDL-CS binding was calculated after subtracting the absorbance values obtained in the absence of CS. Percentages of
inhibition were calculated relative to nM of MDA obtained in PBS, sera or purified IgG from non-immunized rabbits. As controls of specificity were used hR3 mAb or hR3-immunized rabbit sera.

**Inhibition of LDL retention in the artery wall of rats**

The capacity to block LDL retention and oxidation in the artery wall of chP3R99 mAb was evaluated in a rat model described previously. Rats (n=6 per group) were intravenously injected via the tail vein with 1 mg of chP3R99 mAb, hR3 mAb or PBS. After 1h, LDL (4 mg/kg) were intraperitoneally administered, and 24h later the rats were euthanized under anesthesia (80 mg/kg ketamine and 10 mg/kg xylazine HCl, IM) by intracoronary injection of KCl. After perfusion fixing for 5 min with 4% formaldehyde, the aortas were removed and placed in 4% formaldehyde for 24h at 4°C before paraffin embedding.

**Atherosclerosis induction**

Atherosclerosis was induced in NZW rabbits by daily administration of Lipofundin (2 mL/kg, 8 days) through marginal ear vein, as previously described. Nineteen rabbits were subcutaneously injected with 100 µg of chP3R99 mAb in PBS, nine with chP3 mAb, five with chP3R99-LALA and 15 with hR3 mAb (3 doses at weekly intervals) and after the last immunization Lipofundin was administered. As controls, 20 rabbits received PBS before Lipofundin and 15 were injected with PBS both subcutaneously and intravenously. Serum samples were obtained at the beginning of the study, a week after the last immunization and after Lipofundin administration and stored at -20°C until use. The animals were anesthetized with ketamine hydrochloride (35 mg/kg and 5 mg/kg xylazine HCl, IM) followed by intracoronary injection of KCl. Defatted aortic arches were placed in 0.1 mol/L Tris-HCl buffer, pH 7.6, with 1 mmol/L EDTA and 0.2 mmol/L butylated hydroxytoluene and macerated for biochemical determinations. Homogenized tissue was centrifuged at 4500g for 20 min, 4°C and supernatants were stored at -80°C until use. For histopathological studies, the aortas were fixed in 4% buffered formaldehyde (pH 7.4) for 24h and samples from the aortic arch were paraffin-embedded or fixed for electron microscopy analysis. Aortic arches were used for histopathology and redox evaluation, due to the preferential development of Lipofundin-induced atherosclerotic lesions in this segment.
Immunohistochemistry
Paraffin-embedded aortic sections from human atherosclerotic plaques, rats and rabbits were cut into 5-µm slices, deparaffinized, rehydrated, and blocked for endogenous peroxidase activity. Human sections were incubated with biotinylated chimeric mAbs (20 µg/mL) for 30 min at RT, followed by addition of peroxidase-streptavidin complex (1:400; Jackson). Detection of LDL and oxLDL in rat aortas was performed by incubating aortic sections with CBLDL3 and EO6 mAbs (20 µg/mL), respectively, overnight at RT. Then, biotin-conjugated goat anti-murine immunoglobulin antibody (1:400; Jackson) followed by peroxidase-streptavidin complex (1:400; Jackson) was added. To evaluate the accumulation of chP3R99 in rat or rabbit aortas a peroxidase-conjugated goat anti-human IgG antibody was used (1:400, Jackson). Finally, sections were incubated with diaminobenzidine substrate solution and counterstained with hematoxylin. For macrophage detection in rabbit aortas, frozen sections (4 µm) were immunostained with RAM-11 mAb and for CD4 and CD8 lymphocytes detection were immunostained with KEN-4 or 12.C7 mAbs, respectively; followed by the addition of biotinylated goat anti-mouse immunoglobulins antibody. Images of immunostained sections captured digitally with a DP20 camera coupled to an optic light microscope Olympus BX51, were quantified using computerized image analysis (Adobe Photoshop CS3 software).

Histopathology and morphometry
Aortic paraffin-sections from rabbits were stained with Masson’s Trichromic and Alcian Blue staining to detect collagen and GAGs, respectively. Morphometrical analyses were performed to calculate intima-media ratio (IMR) in Verhoeff-Van Giesson stained sections. The sections were analyzed in an optic microscope Olympus BX51. Vascular lesions were classified according to the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association.16

Electron microscopy
For transmission electron microscopy (TEM), samples from rabbit aortic arch were fixed for 1h at 4°C in 3.2% glutaraldehyde, 0.1 mol/L PBS (pH 7.4) and postfixed in 1% OsO4 for 1h. After graded ethanol dehydration, samples were embedded in Spurr low-viscosity epoxy resin for 24h at 37°C. Ultrathin sections were cut into 400-500 Å thick slice with an LKB Ultramicrotome (Nova LKB), counterstained with uranyl acetate and lead citrate, and analyzed in a JEOL JEEM-2000EX.
Lipid parameters

Total serum cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were measured using Randox Ltd. kits.

Redox biomarkers

Redox variables were spectrophotometrically determined in supernatants from homogenates of rabbit aortas and expressed relative to protein content. The superoxide dismutase (SOD) activity was evaluated by using RANSOD Ltd. Kit and catalase (CAT) activity was assessed by following the H$_2$O$_2$ decomposition at 240 nm for 1 min. Glutathione (GSH) was measured as described elsewhere. MDA was determined with a LPO-586 kit (Calbiochem). For peroxidation potential (PP) determination, samples were incubated with 2 mmol/L CuSO$_4$ for 24h at 37°C. PP was calculated by substracting the MDA levels in sera from those obtained 24h after lipid peroxidation induction. Advanced oxidation protein products (AOPP) were measured as described previously and expressed as μmol/L of chloramine-T equivalents. Nitrites/nitrates levels were measured as a surrogate marker of NO content in the samples by the Griess reaction. Nitrates were reduced to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA) followed by Griess reagent in 0.25% phosphoric acid (Sigma). Samples were incubated at RT for 10 min and absorbance was measured at 540 nm in a microplate reader (SUMA).

Statistical analysis

Values were expressed as mean±SEM. Statistical analysis was performed with SPSS 11.5 software. For multiples comparisons one-way ANOVA was used followed by Tukey post-test. Values of $P<0.05$ were considered statistically significant.

Supplemental References


Supplemental Figure I. chP3R99 mAb reactivity to proteins, lipoproteins and phospholipids. ELISA plates were coated with 10 μg/mL of fibronectin (A), laminin (B), collagen I (C), fibrinogen (D), LDL (E), oxLDL (F) or 50 μg/mL of PAPC (G) or POVPC (H). Plates were incubated with mAbs followed by an AP-conjugated goat anti-rabbit IgG. Anti-human fibronectin and EO6 antibodies were used as control. Data are mean±SEM.
Supplemental Figure II. chP3R99 mAb reactivity to atherosclerotic plaque from human aorta. Formalin-fixed paraffin-embedded human aortic sections were incubated with biotinylated chP3R99 (C through I), chP3 (B through H) or the isotype-matched chP3S98 (A through G) mAbs (20 µg/mL). The reaction was detected using a streptavidin-peroxidase complex. Arrows show foam cells (40X). Bars=100-µm.
Supplemental Figure III. Effect of chP3R99 mAb on the oxLDL binding to J774A.1 cells and foam cell formation. (A) Recognition of murine macrophage cell line J774A.1 by chP3 mutants. Cells were incubated with biotinylated chimeric mAbs (10 µg/mL), stained with streptavidin-FITC complex, and analyzed by flow cytometry. (B) Inhibitory effect of chP3R99 mAb on the recognition of J774A.1 cells by an anti-CS antibody. Cells were co-incubated with chimeric (100 µg/mL) and anti-CS (1:100) mAbs, and the latter was detected by adding biotin-conjugated goat anti-murine IgM antibody followed by streptavidin-FITC complex. (C) Inhibition of b-oxLDL binding to J774A.1 cells. Cells were incubated with the chimeric mAbs (100 µg/mL) and b-oxLDL (20 µg/mL) at 4°C for 4h and the latter were detected with the streptavidin-FITC complex. (D) The inhibition of b-oxLDL uptake was performed similar to (C), but cells were incubated with b-oxLDL at 37°C for 4h, permeabilized with 0.1% Triton X-100 and stained with the streptavidin-FITC complex. Data are expressed as the percentage of stained cells of at least two experiments.
Supplemental Figure IV. Effect of chP3R99-LALA mAb on Lipofundin-induced atherosclerosis in NZW rabbits. Hematoxillin & Eosin and RAM-11 stainings (20X) showed intimal thickening (A) and macrophage infiltration (B) in aortas from Lipofundin-treated rabbits. In rabbits treated with chP3R99-LALA no lesions (C) and no macrophage infiltration in the aorta were observed (D). Bars=100-μm.
Supplemental Figure V. Anti-GAG response in rabbits immunized with chP3R99 mAb. ELISA plates were coated with DS (A), heparin (B), HS (C) or hyaluronic acid (D) (10 μg/mL) and incubated with sera from PBS group, Lipofundin-receiving animals or mAbs-IR (1:3000). Also, reactivity to CS of purified non-IR IgG or chP3R99-IR IgG (25 μg/mL) was evaluated. Bars represent the ratio of absorbance values of postimmune to preimmune sera or chP3R99-IR IgG to non-IR IgG.
Supplemental Figure VI. Reactivity of purified IgG from chP3R99-immunized rabbits to proteins from the ECM and LDL. ELISA plates were coated with fibronectin (A), laminin (B), collagen I (C), LDL (D) or oxLDL (E) (10 μg/mL). Purified IgG from non-immunized rabbits (IR) or chP3R99-IR were added (25μg/mL) followed by an AP-conjugated goat anti-rabbit IgG. Data are mean±SEM.