

Cloning and expression of an anti-LDL(-) single-chain variable fragment, and its inhibitory effect on experimental atherosclerosis

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Abbreviations: scFv, single chain variable fragment; nLDL, native LDL; LDL(-), electronegative LDL; *Cd36*, cluster of differentiation 36; *Tlr-4*, toll like receptor 4; *Cox-2*, cyclooxygenase 2

The in vivo modified forms of low-density lipoprotein (LDL) are important for the formation of foam cells and as mediators of the immuno-inflammatory process involved in the progression of atherosclerosis. Electronegative LDL, LDL(-), is a LDL subfraction with pro-inflammatory properties that is present in human blood. To investigate possible atheroprotective effects, an anti-LDL(-) single-chain variable fragment (scFv) was expressed in the methylotrophic yeast *Pichia pastoris* and its activity was evaluated in vitro against macrophages and in experimental atherosclerosis in *Ldlr*^{-/-} mice. The recombinant 2C7 scFv was produced in a yield of 9.5 mg of protein/L. The specificity and affinity of purified 2C7 scFv against LDL(-) was confirmed by ELISA. To assess the activity of 2C7 scFv on foam cell formation, RAW 264.7 macrophages were exposed to LDL(-) in the presence or absence of 2C7 scFv. The 2C7 scFv inhibited the uptake of LDL(-) by macrophages in a dose-dependent manner, and internalization of LDL(-) by these cells was found to be mediated by the CD36 and CD14 receptor. In addition, compared with untreated cells, lipid accumulation in macrophages was decreased, and the expression of Cd36, Tlr-4 and Cox-2 was downregulated in macrophages treated with 2C7 scFv. Importantly, compared with untreated mice, the treatment of *Ldlr*^{-/-} mice with 2C7 scFv decreased the atherosclerotic lesion area at the aortic sinus. In conclusion, our data show that 2C7 scFv inhibits foam cell formation and atherosclerotic plaque development by modulating the expression of genes relevant to atherogenesis. These results encourage further use of this antibody fragment in the development of new therapeutic strategies that neutralize the pro-atherogenic effects of LDL(-).

Introduction

Recombinant monoclonal antibodies (mAbs) are used as therapeutic agents to treat autoimmune and inflammatory diseases because of their high specificity and capacity to function as high-affinity targeting reagents.^{1,2} As of January 2013, 19 mAbs were in Phase 3 clinical trials for non-cancer purposes, including AMG145 and alirocumab for high cholesterol treatment, and an additional 10 mAbs were in Phase 3 studies as treatments for cancer.³ Although widely used for numerous indications, full length mAb therapeutics have disadvantages due to their large size, pharmacokinetics and restricted access to some tissues. Molecular biology strategies thus have been used to generate monovalent antigen-binding (Fab) or single chain variable (scFv) fragments and divalent (e.g., Fab₂¹, diabodies, minibodies) antibody fragments that may also have clinical utility.⁴

The scFv contains the smallest functional unit of the antibody. It is composed of the variable domains of antibody light and heavy chains joined by a hydrophilic and flexible spacer peptide that is 10 to 25 amino acid residues in length.⁴ The antibody binding site is kept intact within the scFv, and there is usually no significant loss of specificity.⁵ Pharmacokinetic properties, however, are changed; for example, scFv are rapidly cleared from the blood and have lower retention time in non-target tissues.⁶ A potential advantage conferred by the small size of the scFv is access to hidden epitope regions where full-length mAbs cannot reach. In addition, the cytotoxicity of scFv is reduced due to their faster removal from the circulation and better disposal of immune complexes that are formed.¹ Because they can be fused with proteins and peptides, the production of scFvs against virtually any important therapeutic target could provide biopharmaceuticals capable of neutralizing key soluble proteins involved in the initiation and progression of diseases such

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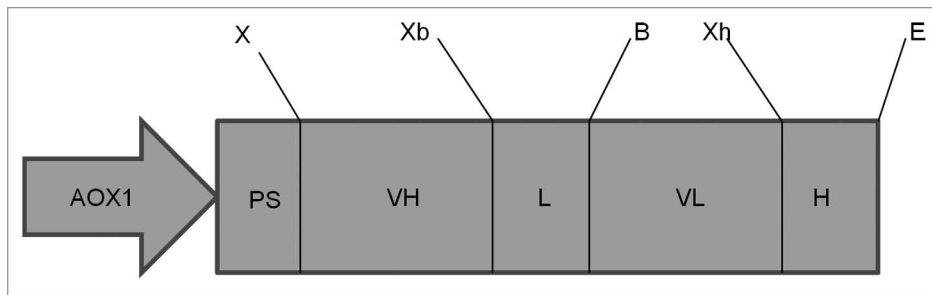


Figure 1. Schematic representation of the 2C7 scFv expression cassette. The scFv expression is driven by the *Pichia pastoris* Alcohol Oxidase 1 promoter. The *Saccharomyces cerevisiae* α -mating type pre-protein leader sequence (PS) is upstream of the 2C7 scFv coding region. The VH gene is flanked by XmaI (X) and Xba I (Xb) restrictions sites. After the linker peptide coding region (L), the VL coding sequence is found in between BglII (B) and Xho I (Xh) sites. A hexahistidine tag (H) is found at the 3' end of the gene followed by a stop codon just before the EcoRI (E) site.

as chronic inflammation and cancer.⁷ The size and simplicity of scFv allow these molecules to be produced in simple heterologous expression systems like *Pichia pastoris*, which is a methylotrophic yeast capable of metabolizing methanol as its sole carbon source that is widely used for high-yield recombinant protein expression.

LDL(-) is an endogenous, minimally modified LDL subfraction found in blood plasma.⁸ Modified forms of LDL are immunogenic and activate both cell-mediated and humoral immune responses, which are pro-inflammatory and likely act in the progression of the chronic inflammatory reaction that is characteristic of atherosclerosis.⁹ The concentration of LDL(-) is elevated in the plasma of patients at high risk for cardiovascular disease as a result of hypercholesterolemia,^{10,11} hypertriglyceridemia,¹² diabetes¹³ or coronary artery disease.^{14,15} LDL(-) has demonstrated pro-inflammatory and pro-atherogenic properties that contribute to the development of atherosclerosis by inducing the recruitment of monocytes to the arterial wall, the secretion of pro-inflammatory mediators by macrophages and endothelial cells, and the induction of autoantibodies.¹⁶ Macrophages retained in the vascular wall accumulate large amounts of modified LDL and become foam cells.¹⁷ Moreover, macrophages produce pro-inflammatory cytokines and participate in functions that integrate the innate and adaptive immune responses during atherosclerosis, including expression of scavenger receptors, such as CD36, and Toll-like receptors (TLRs), such as TLR-4.¹⁸

We previously reported that passive immunization using an anti-LDL(-) mAb in *Ldlr*^{-/-} mice decreased both the cross-sectional area and the number of foam cells in atherosclerotic lesions.¹⁹ In this study, we cloned and expressed an anti-LDL(-) 2C7 scFv in *P. pastoris* and determined its anti-atherogenic activity on 264.7 RAW macrophages and in LDL receptor gene knockout mice (*Ldlr*^{-/-}). Our findings reinforce the potential of novel antibody-based immunotherapeutic approaches that can lead to therapies for complex diseases such as atherosclerosis.

Results

Obtention of the 2C7 scFv. The cDNAs that code for the VH and VL of 2C7 mAb were obtained by reverse transcription polymerase chain reaction using specific immunoglobulin

primer libraries that recognize all VH and VL chain V regions from murine families. The analysis of the sequences in the GenBank and Kabat databanks showed that 2C7 mAb uses a VH segment from Vmu 3.2 (J558) and a Jh4 segment, while VL uses an 8.24/Jk5 segment. The 2C7 scFv was assembled based in the pIg16 vector, a vector for bacterial expression, and then it was subcloned into the *P. pastoris* expression vector pPIgLE, downstream of the AOX1 promoter (Fig. 1). The expression of 2C7 scFv by recombinant *P. pastoris* SMD1168 clone was induced by adding 1% methanol and 0.1 M PMSF every 24 h, at a temperature of

20°C. Under these conditions, we obtained a yield of 9.5 mg/L scFv. The protein was purified by nickel affinity chromatography and two bands were detected in the silver-stained polyacrylamide gels and with western blotting (Fig. 2). The apparent affinity of 2C7 scFv for LDL(-) was assayed by direct ELISA using nLDL as a negative control and 2C7 mAb as a positive control. The results showed that either recombinant 2C7 scFv or mAb were able to bind specifically to LDL(-) (Fig. 3).

Analysis of glycosylation of the 2C7 scFv. The purified 2C7 scFv showed two bands in SDS-PAGE with apparent expected MWs of 30 and 28 kDa, respectively, that were immunoreactive with anti-His antibody. To investigate whether the two purified bands were produced due to hyperglycosylation, the protein was deglycosylated with Endo H. Only one putative N-glycosylation site at CDR-1 of 2C7 scFv light chain was predicted using the BioEdit software. The Endo H-treated material was analyzed by gel electrophoresis and western blotting. The results showed that the deglycosylation treatment of 2C7 scFv converted the two bands into a single band, confirming the predicted glycosylation (Fig. 4).

Detection of negatively charged LDL subfraction in blood plasma of *Ldlr*^{-/-} mice. The anion exchange FLPC chromatography used to separate the LDL subfractions (Fig. 5A) showed three peaks where the first corresponds to the components of the antioxidant cocktail used to prevent oxidation of samples. A second peak corresponds to the native LDL subfraction, similar to the chromatogram of human LDL (Fig. 5B). The third peak contains the LDL subfraction with the highest negative charge (Fig. 5A-B) with a retention time similar to the human LDL(-) subfraction. Thus, the peaks 2 and 3 detected in the fast protein liquid chromatography (FPLC) chromatogram correspond to mouse unmodified LDL (or nLDL) and to LDL(-), respectively. To confirm the identity of the mice LDL subfractions isolated by FPLC, ELISA assays were done with each of these LDL subfractions and compared with nLDL and LDL(-) separated from human LDL by using the 1A3 and 2C7 monoclonal antibodies and the 2C7 scFv, developed by our group. The reactivity profiles of both mouse and human LDL subfractions to the antibodies were similar (Fig. 5C). The reactivity of the 1A3 mAb was lower

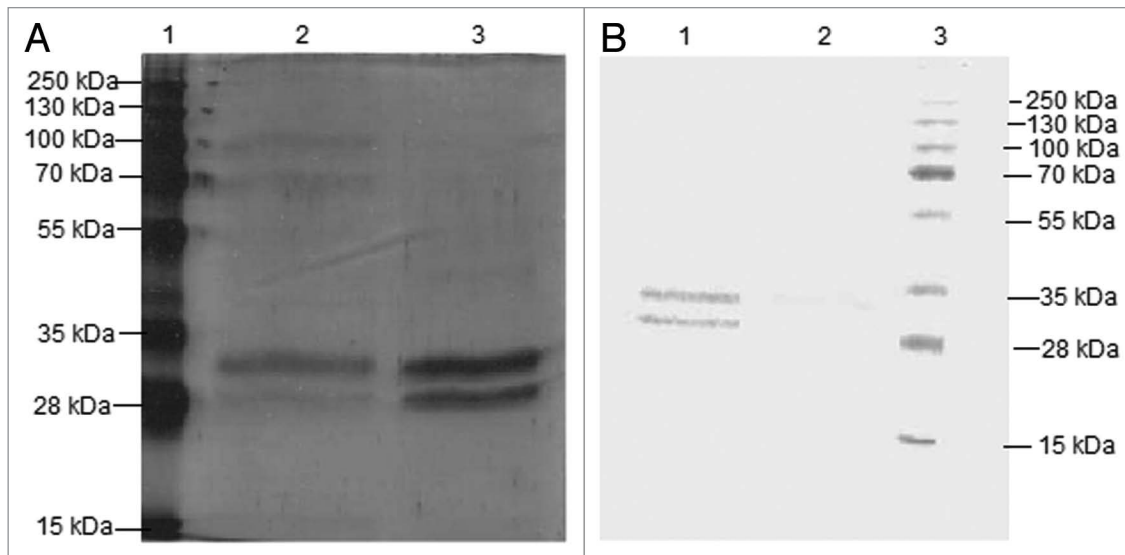


Figure 2. Recombinant protein purification. (A) SDS-PAGE analysis of the protein purified by affinity chromatography from the crude supernatant in line 2 and purified scFv protein from previously concentrated and dialyzed supernatant in line 3. Line 1 corresponds to molecular weight marker. (B) Western blotting analysis. Line 1: purified scFv protein from previously concentrated and dialyzed supernatant. Line 2: purification from the crude supernatant. Line 3: molecular weight marker.

to human and murine LDL(-) compared with the 2C7 mAb and the 2C7 scFv. Thus, the presence of LDL(-) in the LDL fraction of *Ldlr*^{-/-} mice was confirmed by physical chemical and antigenic characteristics.

Macrophage viability. The MTT assay showed that cell viability was not affected in the presence of up to 6.25 $\mu\text{g/mL}$ 2C7 scFv (Fig. 6A). At the highest concentration tested (100 $\mu\text{g/mL}$ 2C7 scFv), cell viability was approximately 60%. In the flow cytometry assays, only 2C7 scFv concentrations higher than 6.25 $\mu\text{g/mL}$ induced death compared with non-treated macrophages (Fig. 6B). The percentage of cell death relative to the log of the concentration of 2C7 scFv is shown in Figure 6C; 50% of total cell death (apoptosis + necrosis) occurred at 29.12 $\mu\text{g/mL}$ 2C7 scFv. At 6.25 $\mu\text{g/mL}$ 2C7 scFv, no significant changes were observed in any stage of the cell cycle in relation to the control (Fig. 6D).

LDL(-) uptake by RAW macrophages. The effect of 2C7 scFv on the formation of foam cells by RAW 264.7 macrophages is shown in Figure 7A. The macrophages incubated with LDL(-) in the presence of 2C7 scFv showed a decrease in intracellular lipid droplets compared with the macrophages treated with LDL(-) in the absence of 2C7 scFv. The semi-quantification of foam cells showed lower LDL(-) uptake by the macrophages when treated with 2C7 scFv compared with untreated cells (Fig. 7B).

Receptor binding studies. To investigate the binding of LDL(-) to RAW 264.7 macrophage receptors, studies were done by flow cytometry and measurement of fluorescence intensity of DIL-labeled LDL(-) to assess the uptake of LDL(-) by macrophages. Figure 8A–F show representative flow cytometry analyses with median fluorescence intensity (MFI) illustrating the autofluorescence of cells. The uptake of LDL(-)-DIL by macrophages (positive control) was inhibited by antibodies reacting with CD36 and CD14 (Fig. 8G). MFI values in Table 1 show

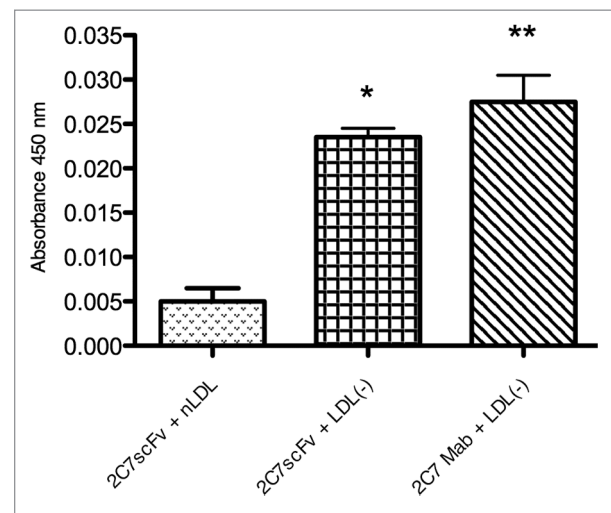


Figure 3. Evaluation of the specificity of 2C7 scFv to LDL(-) by ELISA. 2C7 scFv was added at a concentration of 20 $\mu\text{g/mL}$ to ELISA microplate coated with 1 $\mu\text{g/mL}$ of LDL(-) or nLDL. The microplate was incubated with an anti-His mouse IgG antibody and HRP-conjugated anti-mouse IgG. The absorbance was measured at 450 nm. The results of independent experiments, performed in triplicate, are expressed as the means \pm SEM * $p < 0.05$; ** $p < 0.01$ compared with control; ANOVA followed by the Tukey-Kramer test.

that anti-CD36 and anti-CD14 antibodies were able to reduce the uptake of LDL(-) by macrophages compared with the control. As expected, the anti-TLR4 antibody did not decrease fluorescence intensity compared with control. In cells preincubated with sets of anti-CD36/CD14 and anti-CD14/TLR4 antibodies, there was greater reduction in LDL(-) uptake compared with the incubation of anti-CD36/TLR4 antibodies, which showed higher LDL(-) uptake compared with anti-CD14 antibody alone.

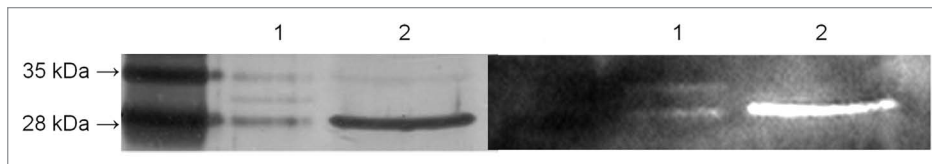


Figure 4. Recombinant protein glycosylation profile. The affinity-purified recombinant 2C7 scFv was treated with Endoglucanase H. The electrophoretic profile was analyzed by SDS-PAGE (left) and western blotting (right) using anti-His IgG Mouse, anti-mouse IgG-HRP and detection with ECL substrate. A protein of one band is observed after endoglucanase treatment (line 2) and compared with the two bands shown in the untreated samples (line 1).

The DIL-labeled LDL(-) uptake by RAW macrophages was decreased by 2C7 scFv in relation to the uptake of DIL-LDL(-) alone (Fig. 9A–C). The higher the 2C7 scFv concentration, the lower the uptake of LDL(-), as shown in Figure 9D. Also, treatment of LDL(-) and 2C7 scFv induced low death in cells by apoptosis and necrosis assays, so the results with only viable cells were demonstrated (Fig. 9E).

Expression of pro-atherogenic genes in macrophages. To understand the mechanisms of action of 2C7 scFv on RAW macrophages treated with LDL(-), the expression of several genes linked to the development of atherosclerosis was analyzed, and the results are shown in Figure 10. The incubation of RAW macrophages with 6.25 µg/mL 2C7 scFv did not induce a significant effect on mRNA expression levels. In contrast, the incubation of macrophages with 37.5 µg/mL LDL(-) induced a statistically significant increase of *Cd36*, *Cox-2* and *Thr-4* mRNA levels. When RAW macrophages were incubated with LDL(-) in the presence of 2C7 scFv, however, significant inhibition of the LDL(-) induced effects on the atherogenic gene mRNA levels was observed.

Effect of 2C7 scFv on experimental atherosclerosis. The atherosclerotic lesions at aortic sinus of *Ldlr*^{-/-} mice treated with 2C7 scFv are shown in Figure 11A. The morphometric analysis of the atherosclerotic plaques demonstrated that the lesion area was significantly decreased ($p < 0.05$) following passive immunization of *Ldlr*^{-/-} mice with 2C7 scFv compared with controls treated with the PBS vehicle (Fig. 11B). The percentages of the atherosclerotic lesion areas of treated groups relative to the control group (vehicle) are represented in Figure 11C. The lipid profile data showed no significant changes of lipid levels among the studied groups (Table 2).

Discussion

In this study, we described the construction, expression and characterization of the recombinant 2C7 scFv antibody fragment and its effect on macrophages and atherosclerotic lesions. Recombinant antibodies, including scFv, are good alternatives for the treatment of various diseases because they are targeted therapeutics that generally show good pharmacokinetics and biodistribution. In addition, their production can be rapid and economical.²⁰

Our 2C7 scFv was expressed in *P. pastoris*, an eukaryotic organism capable of producing secretable soluble proteins with modifications such as disulfide bridges and glycosylation,²¹ and

showed an apparent affinity for LDL(-) only slightly lower than that of the parental 2C7 mAb. This result was expected because scFvs are monovalent, whereas the full length mAb harbors two binding sites for the antigen. Importantly, the 2C7 scFv maintained the same parental antibody specificity for LDL(-), and it is not reactive to native LDL.

Although a 28 kDa protein was detected in the culture supernatant, we observed the presence of two bands with molecular weights of ~28 and 30 kDa in polyacrylamide gels after purification with nickel affinity chromatography. Some studies also reported the expression of two bands of scFv in *P. pastoris*; however, they attributed this to degradation^{22,23} or to incomplete cleavage of the signal sequence.²⁴ Other studies indicate that the additional bands detected may be due to the glycosylation of recombinant proteins with the addition of mannose residues that increase the recombinant protein molecular weight.^{25,26} Yeast can perform glycosylation of the amide nitrogen of asparagine residues in the consensus sequence Asn-X-Thr/Ser, providing N-linked glycosylation. This sequence was found in the 2C7 scFv VL CDR1. The electrophoretic profile of the 2C7 scFv was modified after treatment with Endo H and showed one band. This suggests that the presence of two bands after nickel purification was a result of glycosylation, and not proteolytic degradation.

Wild-type mice contain a low level of cholesterol in the IDL/LDL fraction. *Ldlr*^{-/-} mice, however, show marked increase in the IDL/LDL fraction with high LDL-cholesterol, accompanied by an increase in the amount of apoB-100 and apoE in the plasma.²⁷ In *Ldlr*^{-/-} mice, there is also a reduction in LDL clearance (half-life of 5 h) compared with wild-type mice (half-life of 2 h).²⁷ This increase in the permanence of LDL in blood circulation, combined with the higher LDL level in this animal model, should contribute to the modification of the LDL particles, which allowed their recognition by the 2C7 mAb and scFv, as was observed in the ELISA assay.

The MTT assay showed that glycosylation did not affect the cell viability for 24 h, as the treatment with RAW macrophages was performed for 16 h. Experimental data suggest that glycosylation was not observed in the murine Fab portion derived from anti-LDL(-) mAb because only one band was visualized in polyacrylamide gel (unpublished results). Thus, glycosylation may be a result of the heterologous expression in *P. pastoris*; this did not interfere with scFv binding specificity to LDL(-) or with its *in vitro* biologic activity.

In a cytotoxicity assay using RAW 264.7 macrophages, flow cytometry data showed no induction of either apoptosis or necrosis at concentrations up to 6.25 µg/mL 2C7 scFv. Thus, this concentration was used for further experiments with the macrophages. We previously reported that LDL(-) stimulates the expression of *Cd36*, promoting the accumulation of lipid droplets in the cytoplasm of macrophages and transforming them into foam cells.²⁸ Here, it is clearly shown that 2C7 scFv inhibited

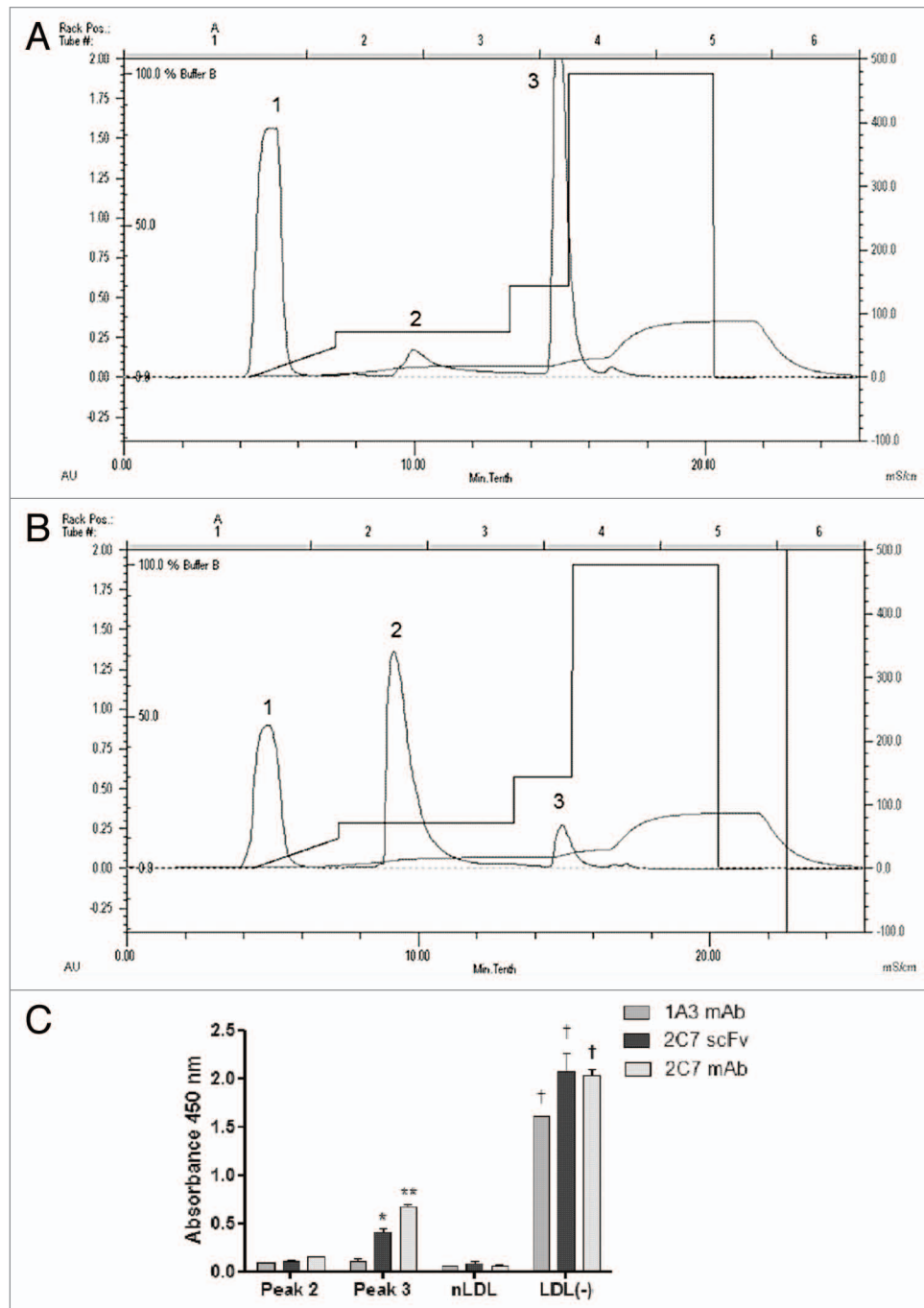


Figure 5. Isolation of LDL(-) from *Ldlr*^{-/-} mice. FPLC chromatographic analysis of mice LDL (A) and human LDL (B), fractionated into peaks 1, 2 and 3. Mice LDL samples were fractionated by anion exchange liquid chromatography based on differences of superficial charges of LDL subfractions. The peak 1 contains components of the antioxidant cocktail used to avoid in vitro LDL oxidation. The reactivity of peaks 2 and 3 to 1A3 and 2C7 monoclonal antibodies and 2C7 scFv were tested by (C) ELISA assays with anti-his and HRP-conjugated anti-mouse antibodies. Absorbance was measured at 450 nm.

LDL(-) uptake by macrophages and downregulated the mRNA expression of *Cd36*. These findings suggest a possible inhibitory action by this recombinant scFv on atherogenesis because it could prevent formation of foam cells in arterial intima. Moreover, 2C7 scFv inhibited the overexpression of pro-inflammatory genes that play an important role in the atherogenic process. We have shown here that LDL(-) induces an upregulation of *Tlr-4* and *Cox-2*

mRNA expression in RAW 264.7 macrophages. In contrast, 2C7 scFv was able to inhibit these LDL(-) actions by blocking the increase of both *Tlr-4* and *Cox-2* mRNA expression. The inhibition of TLR-4 by 2C7 scFv is highly relevant^{29,30} because it has been shown that minimally modified LDL induces the pro-atherogenic activation of macrophages by a TLR-4-dependent mechanism, stimulating the expression of pro-inflammatory

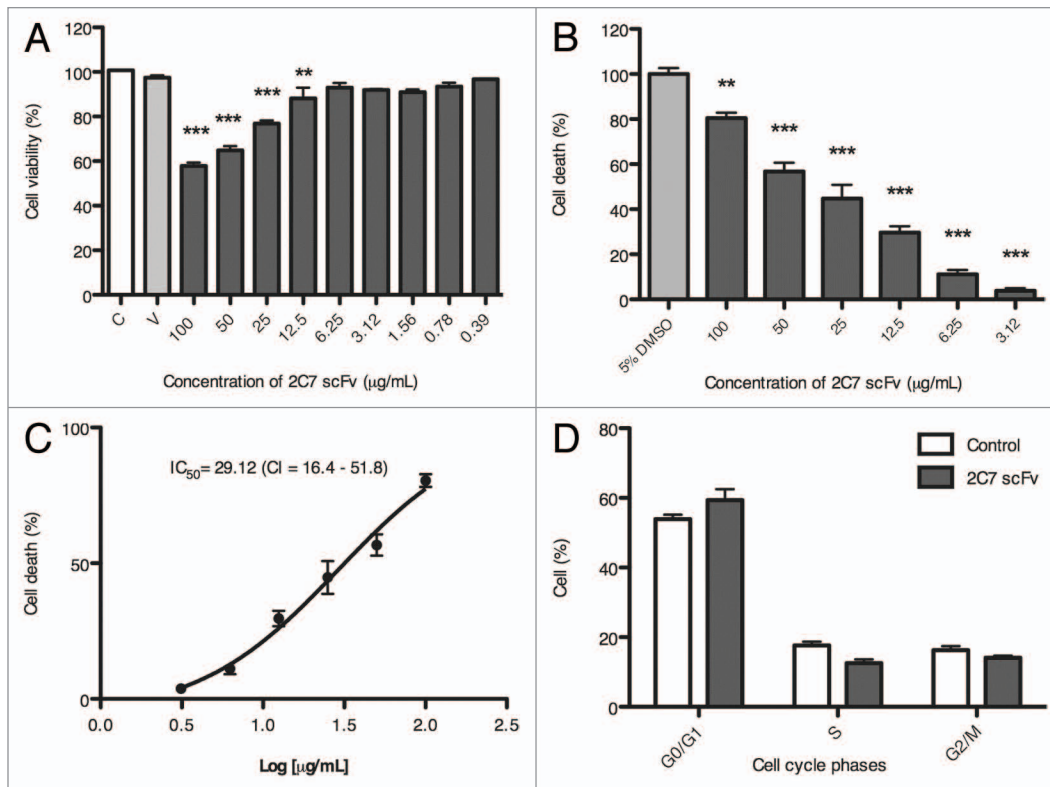


Figure 6. Effect of 2C7 scFv on RAW macrophages. **(A)** Cell viability evaluated by MTT. **(B)** Relative cell death results normalized in relation to DMSO control (100%). **(C)** Percentage of cell death relative to the log of 2C7 scFv concentration. **(D)** Cell cycle data. The results of independent experiments, performed in triplicate, are expressed as the means \pm SEM * p < 0.05; ** p < 0.01 compared with control; ANOVA followed by the Tukey-Kramer test.

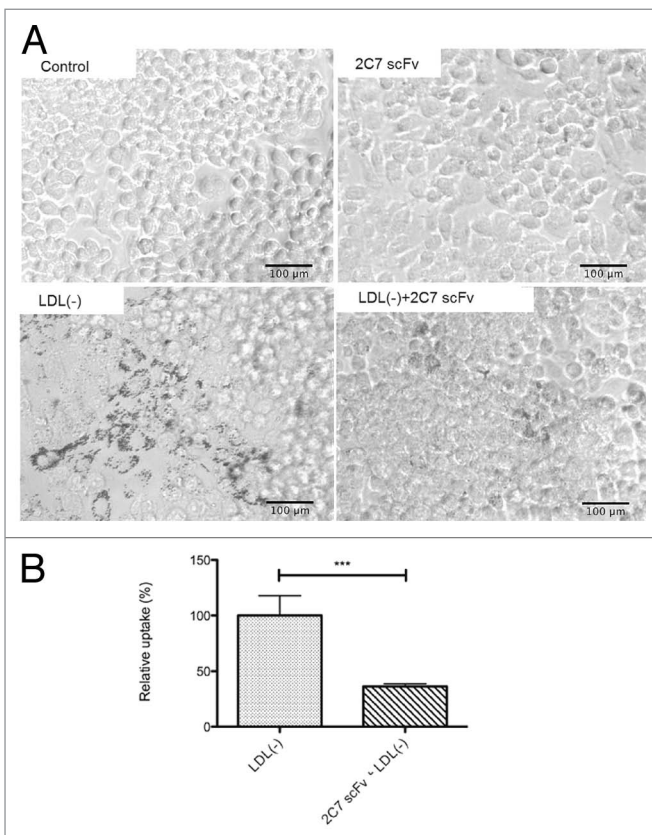


Figure 7. LDL uptake by RAW macrophages. RAW macrophages (10^5 cells/well) were incubated in the presence of LDL(-) and 2C7 scFv for 16 h. **(A)** Representative images show macrophages stained with Oil Red O. Images were obtained using the Motic Images Plus version 2.0 program at a 20 \times magnification. **(B)** Semi-quantification of lipid droplet accumulation in macrophages treated with 2C7 scFv and LDL(-) compared with macrophages treated only with LDL(-). Representative images are from three independent experiments.

cytokines.³⁰ The COX-2 gene is expressed in the foam cell macrophages present in atherosclerotic lesions,³¹ and its overexpression induces the formation of early atherosclerotic lesions in *Ldlr*^{-/-} mice³² and probably in human atherosclerotic lesions.³³ Therefore, the effect of 2C7 scFv on RAW 264.7 macrophages, which promotes the downregulation of *Cox-2*, *Thr-4* and *Cd36* mRNA expression, indicates that this recombinant antibody fragment is able to block the pro-inflammatory and pro-atherogenic actions of LDL(-).

The receptor binding assays done in the present study showed that the entry of LDL(-) in RAW macrophages can occur through CD14 and CD36 receptors, which could be a route by which LDL(-) was able to induce proinflammatory effects on macrophages. In fact, a previous report showed that minimally modified LDL can bind to CD14, making it a likely candidate receptor for LDL(-).²⁹ Recently, a relationship has been established between the increase of CD14 and CD36 expression in circulating human

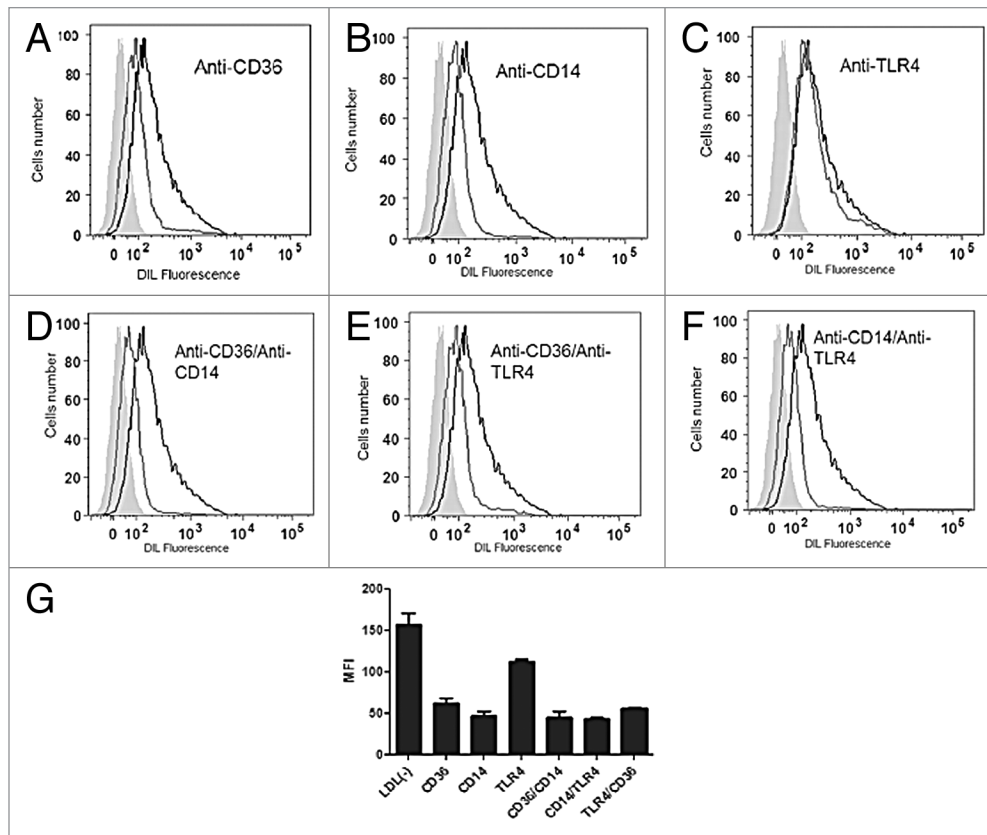


Figure 8. Representative images from flow cytometry analysis of the fluorescence intensity of LDL(-)-DIL taken up by RAW 264.7 macrophages blocked with the following antibodies: (A) anti-CD36, (B) anti-CD14, (C) anti-TLR4, (D) anti-CD36/CD14, (E) anti-CD36/TLR4, (F) anti-CD14/TLR4. (G) Graph showing the decrease of LDL (-)-DIL uptake with blocking antibodies specific to CD36, CD14, and TLR4 receptors. Data are represented as mean of MFI values.

monocytes and the risk of coronary artery disease in patients with cardiovascular disease.³⁴ CD14 is also able to induce the release of pro-inflammatory cytokines in monocytes and macrophages after stimulation by mmLDL.³⁵ We demonstrated that at 6.25 $\mu\text{g}/\text{mL}$ 2C7 scFv reduced the uptake of LDL(-)-DIL by macrophages, and the reduction was greater at higher concentrations of 2C7 scFv. Although cell viability was decreased in the presence of 12.5 and 25 $\mu\text{g}/\text{mL}$ 2C7 scFv, cell viability was unaffected by the co-incubation of LDL(-) and 2C7 scFv at all concentrations used in the flow cytometry analysis. Thus, a dose-dependent effect occurs for the inhibition of LDL(-) uptake by 2C7 scFv.

The atheroprotective action of the 2C7 scFv was confirmed by our studies with *Ldlr*^{-/-} mice. The antibody fragment was able to decrease the atheroma area in the aortic sinus of these animals by approximately 44% with a single weekly dose. Moreover, the atheroprotective action of 2C7 scFv was unrelated to changes in lipid concentrations in blood plasma. Recombinant antibodies against peptides of MDA-modified apoB 100 have been shown to significantly decrease atherosclerosis.³⁶ As previously reported, scFv and Fab against in vitro oxidized LDL inhibited foam cell formation and the progression of atherosclerotic lesions by blocking the binding of oxLDL to macrophages and their subsequent internalization.³⁷ Moreover, passive immunization with anti-tumor necrosis factor and anti-platelet glycoprotein IIb/

Table 1. Fluorescence intensity of LDL(-)-DIL taken up by RAW macrophages in the presence of anti-CD36, anti-CD14 and anti-TLR4 antibodies

Treatment	MFI
LDL(-)	178.5
CD36	83.9
CD14	68.2
TLR4	133.5
CD36/CD14	66.9
CD14/TLR4	64.0
TLR4/CD36	77.1

Values are shown as median fluorescence intensity (MFI) using the treatment of LDL(-)-DIL as control. Treatments with blocking antibodies were compared with the control.

IIIa antibodies have been reported for the treatment of unstable angina and the prevention of restenosis, respectively, as reviewed elsewhere.³⁸

In conclusion, this study, which focused on the production and assessment of a recombinant antibody fragment that recognizes negatively charged LDL particles, showed that 2C7 scFv

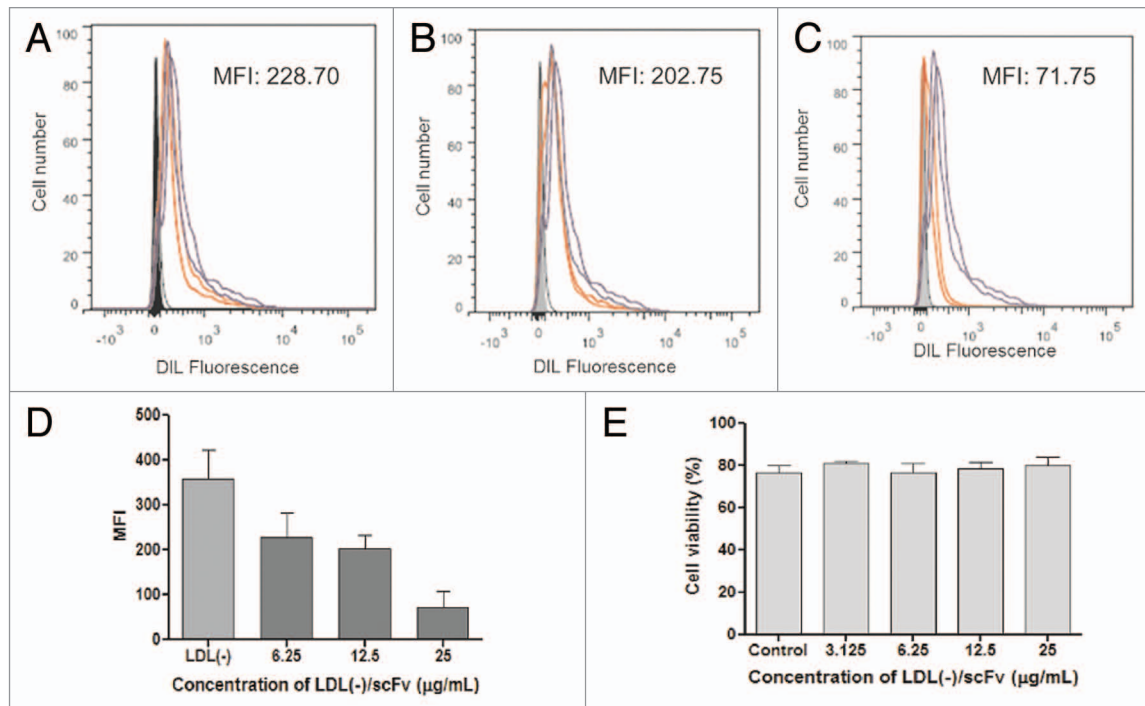


Figure 9. Inhibition of LDL (-)-DIL uptake by different concentrations of 2C7 scFv. The concentrations (A) 6.25, (B) 12.5, and (C) 25 µg/mL were tested. (D) represents quantitative data of uptake inhibition, from the mean of MFI values and (E) cell viability with co-incubation of LDL(-) and 2C7 scFv measured by flow cytometry analysis.

was able to inhibit the formation of macrophage-derived foam cells, the expression of pro-inflammatory factors and the progression of atherosclerosis in *Ldlr*^{-/-} mice. Based on these data, the 2C7 scFv has potential value for future studies on the prevention or treatment of atherosclerosis.

Materials and Methods

Bacteria strains, yeast strains and plasmids. *Escherichia coli* DH5α was used for all plasmid manipulations. SMD1168 strain *P. pastoris* was purchased from Invitrogen Life Technologies (Cat# C17500). For the assembly of the expression cassette, pGEM-T Easy plasmid was purchased from Promega (Cat# A1360). The pIg16 and pPIG16 plasmids were previously described.^{39,40}

Cloning of the 2C7 scFv. The hybridoma 2C7D5F10 (2C7)⁴¹ was cultivated in bottles containing RPMI medium supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin sulfate, 100 U/mL penicillin G sodium and 0.25 µg/mL amphotericin B. The bottles were incubated at 37°C in a 5% CO₂ atmosphere at 95% relative humidity until 10⁶ cells were obtained. To isolate the total RNA, the cells were treated with 1 mL of TRIzol (Cat# 15596-026, Invitrogen Life Technologies) according to the manufacturer's instructions. The cDNAs coding for the antibody variable heavy-chain gene (VH) and the variable light-chain gene (VL) were synthesized using 1 µM each of the primers κ18 (5'-TACAGTTGGT GCAGCATC-3') and 1 (5'-TGGACAGGGA TCCAGAGTTC CAGGTCAC-3') to prepare Cκ and Cγ, respectively. For the amplification of the

VH and VL region cDNA, we used a library of sense primers and the anti-sense primers that were previously described.⁴²⁻⁴⁴ Amplified VH and VL cDNAs were cloned in the pGEM-T Easy plasmid following the manufacturer's instructions. Five clones from each variable region were sequenced in both directions with the T7 (5'-TAATACGACT CATATAGGG-3') and SP6 (5'-GATTTAGGTG ACACTATAG-3') primers using an automatic sequencer MegaBACE 1000 (GE Healthcare) and a DYEnamic ET Dye Terminator Kit (with Thermo Sequenase™ II DNA Polymerase, Cat# US81095, GE Healthcare). For the assembly of murine scFv, the sequences were analyzed by Electropherogram Quality Analysis (available at www.biomol.unb.br/phph/) using the GenBank and Kabat databanks (www.ncbi.nlm.nih.gov/BLAST/).

The murine scFvs genes were assembled using the pIg16 plasmid expression cassette framework.⁴⁵ This plasmid encodes the gene for Z22 scFv fused to the staphylococcal protein A domain (SpA).⁴⁶ The 2C7 VH and VL genes were reamplified using oligonucleotides that created specific restriction sites. The assembly was performed by replacing the Z22 VH and VL genes with the anti-LDL(-) VH and VL genes and by introducing a hexahistidine tag at the 3' terminus of 2C7 VL. This final sequence was inserted into pPIgLE yeast expression vector, a plasmid modified from pPIg 16 vector.

Production of 2C7 scFv in *Pichia pastoris*. *P. pastoris* SMD1168 cells were electroporated with a BTX electroporator model ECM 830, in the presence of linearized plasmid DNA. His⁺ transformants were screened and cultured using the method previously described.⁴⁷ 2C7 scFv was expressed in 200 mL of

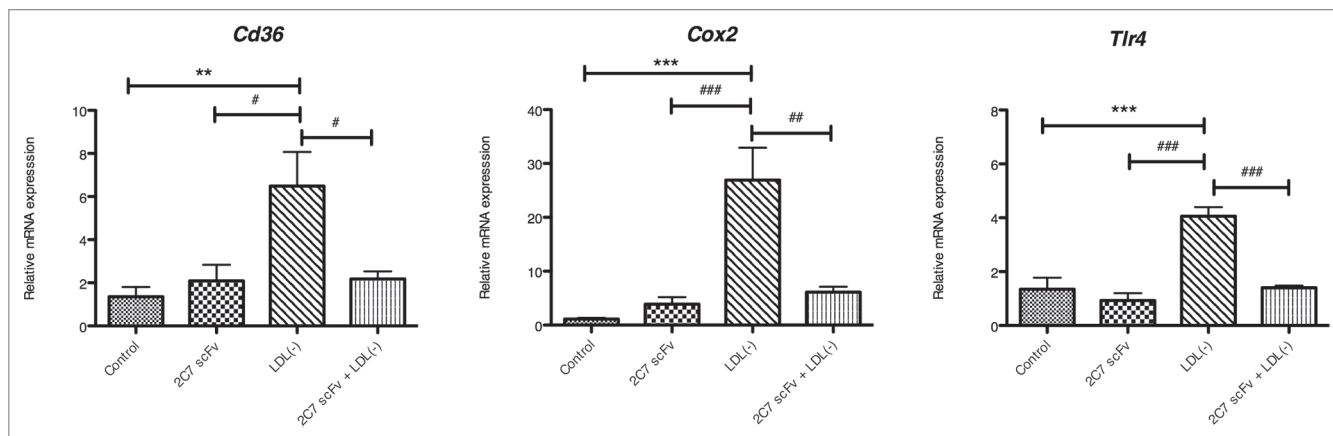


Figure 10. Effect of 2C7 scFv on the relative expression of *Cd36*, *Cox-2* and *Tlr-4* mRNA. Cells were treated with 2C7 scFv (6.25 μ g/mL), LDL(-) (37.5 μ g/ml) or 2C7 scFv + LDL(-) for three hours. The results of independent experiments, performed in triplicate, are expressed as the means \pm SEM * p < 0.05 vs. control; # p < 0.05 compared with treatment with LDL(-); ANOVA followed by the Tukey-Kramer test.

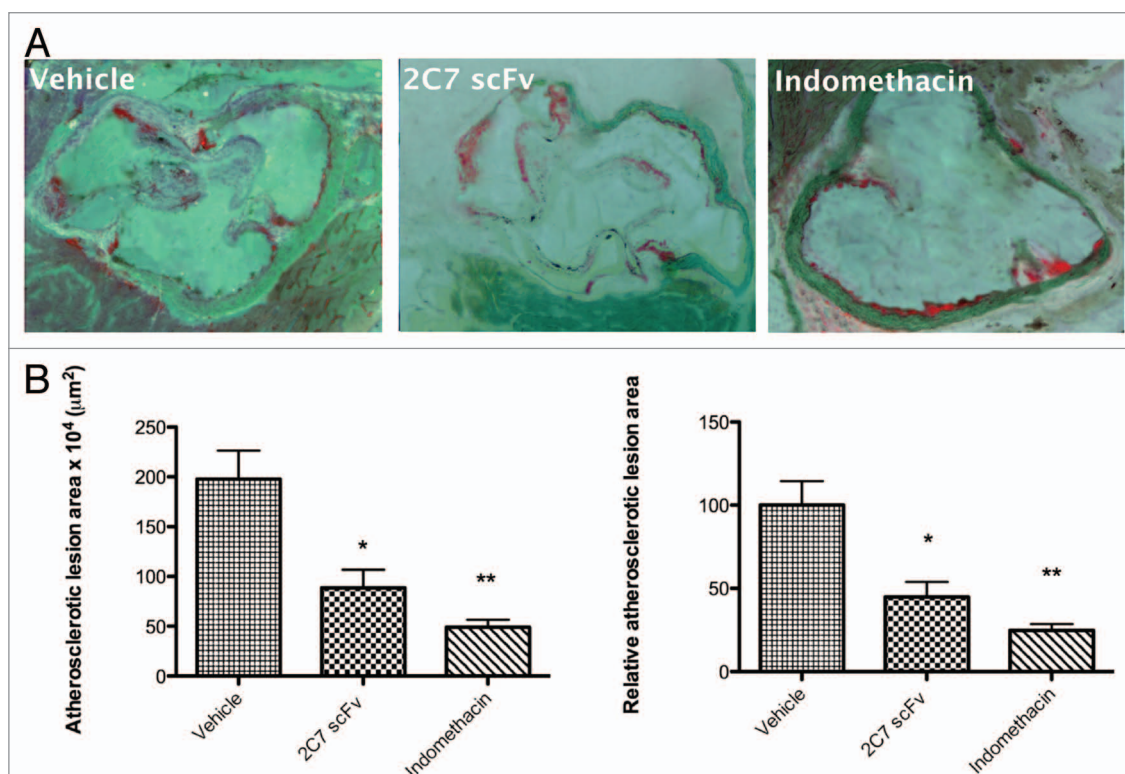


Figure 11. Effect of passive immunization of *Ldlr*^{-/-} male mice with 2C7 scFv on the atherosclerotic lesion development at the aortic sinus. (A) Representative sections of the aortic sinus from the control, 2C7 scFv and positive control groups are shown. Images were obtained using the NIS-Elements AR(tm) version 3.10 at a 10 \times magnification. (B) Mean \pm SEM of atherosclerotic lesion area. (C) Percent of atherosclerotic lesion area in relation to the control. p < 0.05 compared with control; ANOVA followed by the Tukey-Kramer test.

BMGY medium at 30°C at 200 rpm until an OD₆₀₀ of 2–6 was reached. The cells were then centrifuged and resuspended in 200 mL of BMMY medium, with an addition of 1% methanol and 1 mM PMSF every 24 h, and were then incubated for 2 d at 20°C with agitation. The supernatant was harvested by centrifugation, and the cells were resuspended in another 200 mL of BMMY

medium. The culture was incubated for an additional 2 d in the same conditions. The supernatant of the culture was harvested by centrifugation, filtered through a 0.45 μ m filter, and 1 mM PMSF was added. The supernatants were added to 1 mL of Ni Sepharose 6 Fast Flow resin (Cat# 17–5318–01, GE Healthcare). The supernatant (flow through) was decanted, and the resin was poured

Table 2. Lipid profile of *Ldlr*^{-/-} mice after passive immunization with 2C7 scFv

Groups	TC	HDL-C	LDL-C	TG	VLDL-C
Control (PBS)	1860 ± 283	33.4 ± 7.52	1730 ± 267	474.0 ± 113	94.8 ± 22.7
Anti-LDL(-) 2C7 scFv	1630 ± 226	26.3 ± 10.4	1520 ± 209	404 ± 136	80.8 ± 27.1
Indomethacin	1710 ± 314	26.3 ± 4.5	1590 ± 295	465 ± 178	93.0 ± 35.6

The concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG) and very low-density lipoprotein cholesterol (VLDL-C) were determined in the following studied groups: PBS control, 2C7 scFv treatment and indomethacin (positive control). Data are shown in mg/dL as Mean ± S. D. ($p < 0.05$ compared with controls).

into a 1.5 cm × 12 cm (20 mL) Econo-Pac Chromatography column (Cat# 732–1010, Bio-Rad Laboratories). 2C7 scFv was eluted with binding buffer containing 500 mM imidazole. The appropriate fractions were pooled, and the buffer was exchanged with PBS and concentrated using centrifugal filtration devices (Vivaspin MWCO 10,000, Cat# 28–9323–60, GE Healthcare). The purified proteins were separated by SDS-PAGE and then transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare). The membrane was blocked for 16 h with 5% skim milk in PBS at 4°C and subsequently incubated with the following antibodies for 1 h at room temperature: anti-His mouse IgG (Cat# 27–4710–01, GE Healthcare) and anti-mouse IgG-HRP (Cat# A1055, Zymed). The target proteins were detected using an ECL Advance Western Blotting Detection Kit (Cat# RPN2135, GE Healthcare) according to the manufacturer's instructions.

Deglycosylation of 2C7 scFv. For enzymatic deglycosylation, 1 µg of purified 2C7 scFv was denatured with 0.5% SDS and 0.04 M dithiothreitol (DTT) and heated at 100°C for 10 min. It was then added to a reaction buffer (0.5% sodium citrate, pH 5.5) with 1000 units of endoglycosidase H (Cat# P0702S, Endo H, New England Biolabs), which hydrolyzes a single N-acetyl-d-glucosamine (GlcNAc) sugar residue, to cleave high-mannose glycans. The digestion was incubated at 37°C for 16 h and assayed by SDS-PAGE and western blotting as described above.

ELISA assay for 2C7 scFv affinity. The isolation of LDL(-) from human plasma was performed as previously reported.⁴¹ ELISA assays were done according to a previous work⁴¹ with minor modifications including the addition of anti-His mouse IgG (diluted 1:1,000 with 1% skim milk; GE Healthcare) to recognize 2C7 scFv. Specific binding was detected with tetramethyl benzidine (TMB) substrate for color development, and the absorbance was measured at 450 nm. All experiments were approved by the Research Ethics Committee of the Faculty of Pharmaceutical Sciences of the University of Sao Paulo.

Analysis of LDL subfractions from *Ldlr*^{-/-} mice. A pool of blood samples was obtained from *Ldlr*^{-/-} mice treated with hypercholesterolemic diet. Blood was collected with heparinized syringes and the blood plasma was separated by centrifugation. Then, the total LDL fraction was isolated from plasma by ultracentrifugation at 56,000 rpm for 7 h at 4°C. After removing the triglyceride-rich fractions in the supernatant, the infranatant was submitted to a second ultracentrifugation to isolate the LDL fraction. The subfractions of LDL were then separated by FPLC according to the protocol previously described.⁴¹

For the ELISA assay, a 96-well microplate was coated with 10 µg/mL of the following samples: 2 and 3 peaks of FPLC chromatogram of mice samples, human nLDL and LDL(-) for 16 h at 4°C in carbonate-bicarbonate buffer, pH 9.6. After blocking the microplate with 2% milk diluted in PBS, the samples were incubated with 10 µg/mL of 1A3 and 2C7 mAbs and 2C7 scFv for 1 h and 30 min at 37°C. Then, the microplate was incubated with anti-mouse-HRP antibody (diluted 1:1,000 in 1% milk, CAT#1706516, BioRad) for detection with 1A3 and 2C7 mAbs and anti-His (diluted 1:1,000 with 1% milk, CAT#27471001, GE Healthcare) for detection with 2C7 scFv. The binding of samples to the antibodies was evaluated by using TMB as substrate and measuring the absorbance at 450 nm.

Cell culture conditions. Murine macrophages of the RAW264.7 cell line were obtained from the cell bank of the Federal University of Rio de Janeiro (Cat# 0212, UFRJ). RAW 264.7 macrophages were cultured in RPMI media containing 2 mM L-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and 10% fetal bovine serum at 37°C in 5% CO₂ in fully humidified air.

Cell viability, cell death and cell cycle assays. The MTT assay was performed as previously described.⁴⁸ For the apoptosis and necrosis assays (cell death), wells containing 1 × 10⁵ RAW macrophages were treated with different concentrations (3.12 to 100 µg/mL of 2C7 scFv, 12.5 to 62.5 µg/mL of LDL(-) and 37.5 µg/mL of LDL(-) with 3.125 to 25 µg/mL of 2C7 scFv). The cell death and cell cycle assays were performed by flow cytometry. Following 24 h of treatment, the cells were resuspended in the reaction buffer supplied with the kit for the detection of apoptosis and necrosis (APOAF, Cat# A9210, Sigma-Aldrich); 0.625 µg of annexin V - FITC and 2.0 µg of propidium iodide (Cat# P2667, Sigma-Aldrich) were added to the cells according to the manufacturer's instructions. The cells were incubated for 10 min at room temperature, protected from light, and analyzed with a FACSCanto flow cytometer (BD Biosciences). Dimethyl sulfoxide (5%, DMSO, Cat# D8418, Sigma-Aldrich) was used as the positive control for cell death and 10,000 events were observed. For the cell cycle analysis, 2 × 10⁵ cells per well of RAW macrophage were incubated under the same conditions mentioned previously, but the wells were only treated with a concentration of 6.25 µg/mL 2C7 scFv. The cells were lysed with 0.1% sodium citrate and 0.1% Triton, treated with 10 mg/mL RNase A (Cat# 12091–039, Invitrogen Life Technologies) and stained with 1 mg/mL propidium iodide for 30 min, with protection from light, before taking measurements. Data analysis was performed using FlowJo version 9.5.1 software (TreeStar).

LDL uptake assay. The LDL(-) uptake assay for RAW 264.7 macrophages was performed according to previous reports.⁴⁹ Macrophages were exposed to the following treatments: 37.5 µg/mL native LDL (nLDL), 37.5 µg/mL LDL(-) and 37.5 µg/mL LDL(-) plus 6.25 µg/mL 2C7 scFv. Untreated cells were used as the control. The cells were treated for 16 h and evaluated for their level of LDL uptake. The cells were fixed in PBS containing 10% formaldehyde for 30 min at room temperature. Subsequently, the intracellular lipid droplets were stained with Oil Red O (Cat# O0625, Sigma-Aldrich) for 1 h, and their images were obtained with Motic Images Plus 2.0 software (Micro-Optics) for semi-quantification of the foam cells.

Gene expression analysis by qRT-PCR. The LDL uptake assay was used for gene expression analysis. RNA from the treated cells was isolated with TRIzol according to the manufacturer's recommendations. The cDNA was synthesized from 2 µg of total RNA using oligo-dT 12–18 and Superscript III (Cat# 12574–018, Invitrogen Life Technologies). For the real time-PCR reactions, 20 ng of cDNA and specific primers were used. The reactions were performed according to the SYBR Green Master Mix (Cat# 4364346, Applied Biosystems) instructions. The following primers were used: CD36 scavenger receptor (*Cd36*) gene: sense primer, 5'-TTTCCTCTGA CATTTGCAGG TCTA-3', and anti-sense primer, 5'-AAAGGCATTG GCTGGAAGAA-3'; toll-like receptor-4 (*Tlr-4*): sense primer, 5'-TCATGGCACT GTTCTTCTCCT-3' and anti-sense primer, 5'-CATCAGGGAC TTTGCTGAGT T-3'; cyclooxygenase-2 (*Cox-2*) enzyme: sense primer, 5'-TGGTGCCTGG TCTGATGATG-3' and anti-sense primer, 5'-GTGGTAACCG CTCAGGTGTT G-3' and 18S rRNA: sense primer, 5'-GTAACCCGTT GAACCCCAT-3' and anti-sense primer, 5'-CCATCCAATC GGTAGTAGCG-3'. The expression levels of mRNA were evaluated by the $\Delta\Delta C_t$ method.⁵⁰

1,1'-diododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DIL) labeling of LDL(-). One mg of LDL(-) was incubated with 150 µg of DIL (CAT#D282, Life Technologies) diluted in 2 mL of lipoprotein deficient serum⁵¹ and this mixture was incubated at 37°C for 8 h. After incubation, the mixture was separated by ultracentrifugation at 56,000 rpm for 7 h at 4°C to separate the LDL(-) from the excess of free DIL. LDL(-)-DIL was dialyzed against PBS and quantified by BCA method (CAT #23225, Thermo Scientific).

Receptors binding studies in macrophages. For binding studies, 10×10^5 macrophage cells were plated per well and 21 h later the cells were pre-incubated with 10 µg/mL of blocking antibodies against CD36 (CAT#Ab78054, Abcam), CD14 (CAT#Ab78313, Abcam) and TLR-4 (CAT#Ab47093, Abcam) receptors. After 3 h, 37.5 µg/mL LDL(-)-DIL was added to the cells and maintained for 16 h as mentioned for cell culture conditions described in the Materials and Methods section. To measure the inhibition of LDL(-)-DIL uptake, RAW macrophages were treated with a predetermined concentration of 37.5 µg/mL LDL(-) and varying concentrations of 2C7 scFv (6.25, 12.5 and 25 µg/mL) for 16 h. The medium was then removed and cells were detached from the plate using cold PBS and centrifuged at 1500 rpm for 5 min. The cells were washed 2 times with PBS.

Finally, cells were resuspended in 200 µL of PBS and the fluorescence of LDL(-)-DIL was determined by flow cytometry. The signals from DIL were shown in a logarithmic fluorescence intensity, expressed as the difference in the MFI captured from cells treated with blocking antibodies or 2C7 scFv compared with cells treated only with LDL(-)-DIL.

Animals, chow and experimental design. Male C57BL/6J homozygous LDL receptor-deficient mice (*Ldlr*^{-/-}) were purchased from Jackson Laboratory (Bar Harbor). The animals were maintained in individual cages at 22°C on a 12 h light–dark cycle. A total of 24 *Ldlr*^{-/-} mice (n = 8 per group, 12 weeks old) were divided into three groups and were intravenously administered a single dose per week of one of the following: vehicle (PBS), 2C7 scFv (5 mg/kg of body weight) and anti-inflammatory positive control (indomethacin, 1 mg/kg of body weight). The experiments were performed using an initial atherosclerotic lesion protocol as previously described.¹⁹ All mice were fed a semi-synthetic chow that was based on a Western-type diet containing 20% fat, 0.5% (w/w) cholesterol (Sigma-Aldrich), 0.5% (w/w) colic acid (Sigma-Aldrich), 16.5% casein, vitamins and minerals according to the recommendations of American Institute of Nutrition (AIN)-93.⁵² All procedures were approved by the Ethics Committee for Animal Studies of the Faculty of Pharmaceutical Sciences, University of Sao Paulo in agreement with the guidelines of the Brazilian College for Animal Experimentation.

Biochemical assessment of serum lipid profile. After treatment, mice were anesthetized with xylazine hydrochloride (2.0 g/100 ml; Vetbrands) and ketamine hydrochloride (1.0 g/10 ml; Vetnil) at doses of 5 mg/Kg and 10 mg/kg, respectively, and blood was collected by cardiac puncture. The blood samples were then centrifuged at 1500× g for 15 min at 4°C to obtain serum. The mice serum was utilized for determination of lipid profile [total cholesterol, triglyceride, cholesterol high-density lipoprotein (HDL-C), cholesterol low-density lipoprotein (LDL-C) and cholesterol very low-density lipoprotein (VLDL-C)]. All determinations were done with commercial kits from Labtest Diagnóstica, by direct methods without previous treatment of the samples. The results of the lipid profile were expressed in mg/dL.

Preparation of histological sections and measurement of atherosclerotic lesion area. The preparation of histological sections and the measurement of atherosclerotic lesion area were performed as previously reported.⁵³ The inclusion of the tissue for slicing was performed in three solutions of different concentrations of gelatin: initially 5% solution of gelatin for three hours, then a 10% solution for three hours and finally a 25% solution for 16 h, with all steps occurring in a water bath (temperature between 40–50°C). The ventricles were sectioned from the apex and base in a plane parallel to a line defined by the edges of the lateral atria. Consecutive cuts with 6 mm thick were collected between the aortic sinus and the aortic onset with an average length of 250–300 microns.⁵⁴ The slides were stained with Oil Red-O (Sigma) and the sections were analyzed by Nikon optical microscope coupled to a camera for image capture program performed by the NIS-Elements AR (tm) version 3.10. To obtain the area of the lesions and quantify atherosclerotic lesions close

to the aortic valve in the aortic root, Axio Vision® was used for image acquisition, processing and analyses of all histological sections. All analyses were double-blind and were performed independently by two observers.

Statistical analysis. Results were expressed as the means ± SEM. The mRNA expression data were analyzed using ANOVA and Student t-tests. Tukey's test was used for the analysis of paired comparisons. All calculations were performed using GraphPad Prism software. A value of $p < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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