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GEINDRE, Sylvie Françoise, et al.

Abstract
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Reference

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Immunization of LDL receptor-deficient mice with β₂-glycoprotein 1 or human serum albumin induces a more inflammatory phenotype in atherosclerotic plaques

Sylvie Dunoyer-Geindre¹, Brenda R. Kwak², Graziano Pelli², Isabelle Roth², Nathalie Satta¹, Richard J. Fish¹,², Guido Reber¹, François Mach²,³, Egbert K. O. Kruithof¹,³, Philippe de Moerloose¹,³

¹Service of Angiology and Hemostasis and ²Service of Cardiology, Department of Internal Medicine, University Hospital, and the ³Faculty of Medicine, University of Geneva; Geneva, Switzerland

Summary

Antiphospholipid antibodies are a risk factor for venous and arterial thrombosis and may contribute to the development of atherosclerosis. The aim of this study was to investigate whether antibodies to human β₂-glycoprotein 1 (β₂GP1), as a model of antiphospholipid antibodies, modify the phenotype of atherosclerotic lesions. LDL receptor-deficient mice were immunized with human β₂GP1, human serum albumin (HSA), or not immunized, and fed a high-cholesterol diet for 14 weeks. Some mice also received pravastatin. Immunization with human β₂GP1 or HSA resulted in formation of autoantibodies recognizing murine β₂GP1 or murine albumin, respectively. We quantified atherosclerotic lesion development and mRNA levels of inflammation-associated proteins in the thoraco-abdominal aorta as well as lesion development, cellular composition and collagen content in the aortic roots. Immunization with β₂GP1 or HSA had no effect on lesion size, but modified the expression in plaque areas of several inflammation-associated proteins. Expression of matrix metalloproteinase-9, tissue factor, interferon-gamma and CD25 was highest in the thoraco-abdominal aorta of β₂GP1-immunized mice, lowest in non-immunized mice and intermediate in HSA-immunized animals. Immunization with β₂GP1, but not HSA, resulted in a lower smooth muscle cell and collagen content of lesions in aortic roots. Statin treatment partially reversed the effects of β₂GP1 immunization. We conclude that immunization with β₂GP1, and to a lesser extent with HSA, leads to modifications in the cellular and protein composition of atherosclerotic plaques, which are associated with a more inflammatory phenotype. Statin treatment partially prevents these changes.

Keywords
Antiphospholipid antibodies, atherosclerosis, β₂-glycoprotein 1, immunization, statins

Animal Models

Introduction

Atherosclerosis is an inflammatory disease modulated by genetic and environmental factors. Immune mechanisms may be implicated at different stages of atherosclerotic plaque development and progression. Autoimmune diseases, such as systemic lupus erythematosus, have been associated with an increased risk of cardiovascular disease (1). The antiphospholipid syndrome is an autoimmune disease characterized by arterial or venous thromboembolism, and/or obstetric complications (including recurrent fetal loss) and the presence of antiphospholipid antibodies (aPLA) (2). Several studies suggested that aPLA contribute to the development of atherothrombosis (3, 4), and increased levels of aPLA have been detected in young patients with severe atherosclerosis (5). Furthermore, elevated aPLA was associated with an increased intima-media thickness of the carotid artery (6). Different mechanisms have been proposed to explain the association of aPLA with atherosclerosis. The major target of aPLA, β₂GP1, is present in human atherosclerotic plaques (7). aPLA enhance the uptake of oxidized LDL by macrophages (8). In addition, aPLA are known to activate endothelial cells and monocytes (9, 10), leading to increased expression of tissue fac-

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Correspondence to:
Philippe de Moerloose
Service of Angiology and Hemostasis
University Hospital of Geneva
24, Rue Michel-du-Crest
1211 Geneva 14, Switzerland
Tel.: +41 22 37 29 751, Fax: +41 22 37 29 777
E-mail: philippe.demoerloose@hcuge.ch

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tor (TF), of chemokines and of leukocyte-adhesion molecules. Such aPLA-mediated cell activation may induce a pro-inflammatory state that is known to promote atherosogenesis (11, 12).

Studies in an atherosclerosis animal model support the hypothesis that aPLA have a proatherogenic effect. Indeed, immunization of LDL receptor (LDLR)-deficient mice with human β2GP1 fed a normal diet resulted in acceleration of aortic fatty-streak formation (13). Similarly, an enhanced early atherosclerosis development was observed in apolipoprotein E (apoE)-deficient mice immunized with human β2GP1 and fed a high-fat diet. This effect was not associated with an increased susceptibility of LDL to ex-vivo oxidation (14) but may be due to an autoimmune response manifested by the generation of autoantibodies directed against murine β2GP1 (15). In addition, transfer of β2GP1-reactive lymphocytes promoted fatty-streak formation in these mice, providing additional evidence for the implication of autoimmune mechanisms in atherogenesis (16).

Statins are lipid-lowering drugs and are widely used in the treatment of atherosclerosis. They exhibit pleiotropic effects including anti-inflammatory and immunomodulatory properties (17). In a murine aPLA-induced thrombosis model, treatment with fluvastatin resulted in a decreased thrombus size and diminished leukocyte adhesion to the vascular wall (18).

Clinically, a major determinant of the risk of myocardial infarction is not the degree of stenosis, but rather the stability of the atherosclerotic plaques (11). Histological studies have established that vulnerable plaques exhibit a thin fibrous cap, a lipid-rich necrotic core, contain numerous inflammatory cells and have a relatively low number of smooth muscle cells. Studies in apoE-deficient mice, on a prolonged lipid-rich diet, have shown that plaque rupture occurs in murine models of atherosclerosis, and that the ruptured plaques display many features of unstable plaques in human arteries (19).

The aim of this study was to investigate to what extent the generation of anti-β2GP1 antibodies induced by β2GP1-immunization influenced atherosclerotic plaque development and plaque composition in LDL-deficient mice fed a long-term high-fat diet. Immunization with human serum albumin was used as a control for potential non-specific autoimmune effects. In addition, we investigated the effect of pravastatin treatment on plaque development and composition.

## Material and methods

### Animals

Two experimental series were completed: one for analysis of atherosclerotic lesion size in the abdominal aorta, and quantification and immuno-analysis of the root sections (36 mice), and one for gene-expression analysis in the abdominal aorta (28 mice). Ten-week-old female LDLR-deficient mice C57BL/6J were immunized subcutaneously with human β2GP1 (a kind gift of Dr. Alain Bosseloir, Zentech, Angleur, Belgium) or human serum albumin (Galexis SA, Ecublens, Switzerland) both in RIBI™ adjuvant (Sigma, Saint Louis, MO, USA). Ten µg of antigen was injected per mouse and a 10-µg boost was injected 15 days later. A control group with the same genetic background and derived from the same litters was not immunized. Ten days after the boost, mice were weighed, and blood was obtained from the retro-orbital plexus. All the mice were fed a high-cholesterol diet (1.25% cholesterol, 0% cholate, product #D12108; Research Diets, New Brunswick, NJ, USA) for 14 weeks. Half of the immunized mice received pravastatin at 10 mg kg\(^{-1}\) day\(^{-1}\) in the drinking water. The mice were allowed access to food and water ad libitum. Attribution to the different immunization and pravastatin-treatment groups was done randomly.

After 14 weeks, mice were sacrificed, blood samples taken, and aortas perfused with 0.9% NaCl. The thoraco-abdominal aorta was fixed in 2% paraformaldehyde or frozen in liquid nitrogen for mRNA extraction. The aortic roots were snap-frozen in optimal cutting temperature (OCT) compound (Microm, Walldorf, Germany) for cryosectioning.

The experiments were performed according to institutional guidelines, and the study protocol was approved by the local veterinary authorities and ethics commission.

### Anti-β2GP1 or anti-albumin antibody detection

Antibodies to β2GP1, human serum albumin (HSA) and murine serum albumin (MSA) were analysed by ELISA in sera of all mice at the beginning of the diet (10 days after the boost) and after sacrifice. ELISA microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with human β2GP1 (15 µg/ml in phosphate-buffered saline [PBS]) (Hyphen Biomed, Andresy, France), HSA or MSA (Sigma) (both 50 µg/ml in carbonate buffer).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>β-actin</td>
<td>5'-TTGTTGCCGGGTCCACA-3'</td>
<td>5'-ACCAGCCGCGGAAATTCG-3'</td>
</tr>
<tr>
<td>CD68</td>
<td>5'-TTAATTGACACACTTATGTG-3'</td>
<td>5'-ACGAAAGGCAAGAGGGAAGACACAC-3'</td>
</tr>
<tr>
<td>CD4</td>
<td>5'-AGATACAGCTTCTCAGGATGATG-3'</td>
<td>5'-TTGACGACGGAAGACACAC-3'</td>
</tr>
<tr>
<td>CD25</td>
<td>5'-TCTCCTGGCAGGACGGTCTC-3'</td>
<td>5'-AACGCTACACACTGCTACATGTGG-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-AAGCTACACACTGCTACATGTGG-3'</td>
<td>5'-ACGAAAGGCAAGAGGGAAGACACAC-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-CAGCAAGGCGAGGAGGGAAGACACAC-3'</td>
<td>5'-TCTCCTGGCAGGACGGTCTC-3'</td>
</tr>
<tr>
<td>TF</td>
<td>5'-GAGAAAGGCAAGAGGGAAGACACAC-3'</td>
<td>5'-ACGAAAGGCAAGAGGGAAGACACAC-3'</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-CCAGCGGCAACTGCTACATGTGG-3'</td>
<td>5'-ACGAAAGGCAAGAGGGAAGACACAC-3'</td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-CAGCAAGGCGAGGAGGGAAGACACAC-3'</td>
<td>5'-GAGAAAGGCAAGAGGGAAGACACAC-3'</td>
</tr>
<tr>
<td>IL 10</td>
<td>5'-GAGAAATCGATGACACGC-3'</td>
<td>5'-GAGAAATCGATGACACGC-3'</td>
</tr>
</tbody>
</table>

Table 1: Primers used for quantitative RT-PCR.
buffer pH 9.6). The plates were incubated for 2 hours (h) at room temperature with serum samples (1:50 diluted in PBS). The presence of IgG against human β2GP1, HSA or MSA was detected using horseradish peroxidase-conjugated goat anti-mouse IgG. The presence of IgM against β2GP1 was analysed according to the same protocol and detected using alkaline phosphatase-conjugated goat anti-mouse IgM. Colour was developed by adding the appropriate substrate (R&D Systems, Abingdon, UK). Absorbances were measured at 450 or 490 nm, respectively.

The presence of autoantibodies to mouse β2GP1 was analysed by sandwich ELISA. The assay took advantage of the immunological cross-reactivity between human and mouse β2GP1 (15). ELISA microtiter plates (Maxisorp, Nunc) were coated with immunopurified rabbit anti-human β2GP1 (2 μg/ml in carbonate buffer pH 9.6) (Hyphen Biomed). The plates were blocked for 2 h with 0.6% gelatine in PBS. The mouse serum samples (diluted 1:50 in PBS containing 0.3% gelatine, 0.05% Tween 20) were incubated for 2 h in these plates. In this approach the mouse β2GP1 contained in the serum samples is captured by the coating antibodies and retains the autoantibodies directed against mouse β2GP1. The amount of mouse IgG bound to the plates was quantified using horseradish peroxidase-conjugated goat anti-mouse IgG.

**Lipid analysis**

Mouse plasma cholesterol concentrations were determined for all the animals at the end of the experimental period as previously described (20).

**Assessment of atherosclerotic lesion development**

The extent of atherosclerosis in the thoraco-abdominal aorta and in the aortic roots was assessed according to standardized methods (21). In short: the thoraco-abdominal aorta was opened longitudinally, and the lesion areas in en-face preparations were stained with Sudan IV. The percentage of surface with lipid deposits was calculated by dividing the stained area by the total aorta surface. Sections of the roots were stained with Sudan IV. Average lesion area from six sections 50 μm distant from each other was calculated. The position of the analysed sections within the roots was standardized for all animals. Lipid stained areas were determined using MetaMorph 6 Software (Feldbach, Switzerland).

**Immunohistochemistry of atherosclerotic lesions**

Masson’s trichrome staining

To determine the smooth muscle and collagen content of the fibrous cap, 5-μm cryosections of the aortic roots were fixed with Bouin’s solution and stained with Masson’s trichrome reagent.

**Immunohistochemical analysis**

This was done on ice-cold acetone-fixed cryosections. After fixation the sections were washed in PBS, blocked in blocking buffer (10% normal goat serum in PBS 0.1%Tween 20), washed in PBS and incubated for 90 minutes (min) with primary antibodies in blocking buffer. Rabbit anti-mouse Mac 3 (BD Pharmingen, San Diego, CA, USA) was used for macrophage quantification, rabbit anti-mouse smooth muscle myosin heavy chain (sm-MHC, Biomedical Technologies, Stoughton, MA, USA) for smooth muscle cell (SMC) quantification and rat anti mouse CD4 (BD Pharmingen) for T-cell quantification. The sections were incubated for 45 min with biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-rat IgG, respectively (Vector Lab, Burlingame, CA, USA). The presence of secondary antibodies was revealed using an avidin-biotin-alkaline phosphatase substrate kit (Vector Lab). Slides were counterstained with hemalun (Sigma). Specificity was confirmed by omitting the primary antibody. Collagen was stained with 0.1% Sirius red in saturated picric acid. Quantitative analysis of stained areas was performed with the aid of MetaMorph 6 Software. The areas were normalized by dividing the area of specific immunostaining by the total plaque area stained with Sudan IV in the proximate tissue slice.

**RNA extraction and reverse transcription**

Total RNA was extracted from frozen thoraco-abdominal aortas using Tri Reagent (MRC Inc, Cincinnati, OH, USA), according to the manufacturer’s instructions. The RNA yield and purity were assessed by absorbance measurements at 260 and 280 nm. From each sample, 1 μg of RNA was used as template for cDNA

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**Figure 1: Antibody binding in sera of mice immunized with human β2GP1 or human serum albumin (HSA) after a 14-week diet period.** IgG binding to human or murine β2GP1 was detected in β2GP1-immunized mice and in control mice. IgG binding to human or murine serum albumin was determined in HSA-immunized mice and control mice. The results are shown separately for mice treated with pravastatin or not and represent means ± SEM. The groups of mice were: Control (CT) (n = 12), HSA – pravastatin mice (n = 12), HSA + pravastatin mice (n = 15), β2GP1 – pravastatin mice (n = 13), β2GP1 + pravastatin mice (n = 12).
synthesis using the ImPromII™ reverse transcription system (Promega, Madison, WI, USA). Negative controls were processed in parallel without reverse transcriptase.

**Quantitative real-time reverse transcriptase PCR assay**
Relative mRNA levels were quantified by real-time PCR, using cDNA templates and an Applied Biosystems Prism 7000 instrument and SYBR-green® PCR master mix reagent (Applied Biosystems, Warrington, UK). Data were analyzed using the ΔC_T method and Applied Biosystems Prism software and β-actin as the housekeeping-gene control. The forward and reverse primers for all PCRs are given in Table 1.

**Statistical analysis**
Data are presented as mean ± standard error (SEM). Data sets containing multiple groups were analysed by ANOVA followed by the non-paired Student t-test. Differences were considered statistically significant at a p-value of <0.05.

**Results**

**Analysis of antibody and cholesterol levels**
Mice were immunized with either human β2GP1 or HSA. We analysed whether mice developed antibodies directed against the injected antigen (human β2GP1 or HSA) and also whether mice developed autoantibodies to murine β2GP1 or MSA. Results are given in Figure 1. Immunization of mice with human β2GP1 resulted in the generation of anti-human β2GP1 IgG that were detectable both 10 days after the boost injection, at the start the cholesterol-rich diet (not shown) and also at the end of the 14-week diet period (Fig. 1). We were unable to detect anti-human β2GP1 IgM in any of the samples. In all mice immunized with human

<table>
<thead>
<tr>
<th>Group</th>
<th>Cholesterol after diet (mg/dl)</th>
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<tbody>
<tr>
<td>Control</td>
<td>2223 ± 242</td>
</tr>
<tr>
<td>HSA no prava</td>
<td>2198 ± 210</td>
</tr>
<tr>
<td>HSA + prava</td>
<td>1743 ± 11</td>
</tr>
<tr>
<td>β2GP1 no prava</td>
<td>2288 ± 178</td>
</tr>
<tr>
<td>β2GP1 + prava</td>
<td>1811 ± 327</td>
</tr>
</tbody>
</table>

HSA, human serum albumin; prava, pravastatin; β2GP1, β2-glycoprotein I.
β₂GP1, autoantibodies to murine β₂GP1 were detectable at the end of the 14-week diet period (Fig. 1).

All mice immunized with HSA had high levels of anti-HSA IgG antibodies and developed antibodies that cross-reacted with MSA (Fig. 1). We observed no difference in antibody levels between the non-treated and the pravastatin-treated animals. Cholesterol levels were measured in the sera of all animals at the end of the experimental period.

No differences were observed between the control mice and the mice immunized with β₂GP1 or HSA. However, pravastatin treatment resulted in a 21% reduction in cholesterol levels both in the β₂GP1-immunized and HSA-immunized mice (Table 2).

Quantification of atherosclerotic lesion development
The extent of atherosclerotic lesion development was analysed in the thoraco-abdominal aorta and in the aortic roots after 14 weeks of high-cholesterol diet. We observed that lesion development was similar between β₂GP1-immunized mice, HSA-immunized mice and non-immunized control mice, whereas pravastatin-treatment significantly reduced lesion development in the thoraco-abdominal aorta for both β₂GP1-immunized (p<0.05) and HSA-immunized groups (p<0.01) (Fig. 2A). Likewise for the roots, no difference was observed in lesion development between β₂GP1-immunized mice, HSA-immunized mice and non-immunized control mice. Pravastatin treatment reduced lesion size in the mice immunized with either β₂GP1 or HSA. Differences, however, were not statistically significant (p=0.08) (Fig. 2B).

Quantitative immunohistochemical analysis of lesions in the aortic roots
We analysed the lesions in the aortic roots by Masson’s trichrome staining, which stains the collagen and smooth muscle cells composing the fibrous cap, whereas the lipid pool appears as empty spaces in stained sections (Fig 3). A quantitative analysis of the results showed no significant differences in the ratio between the fibrous cap and lipid core area between the various immunization and control groups (data not shown). Quantitative immunohistochemical analysis for collagen, macrophages, SMC and CD4 were made on aortic roots and normalized to the total lesion areas. The collagen content was significantly lower in β₂GP1-immunized mice than in the HSA-immunized or control mice. Pravastatin treatment had no effect on the collagen content (Fig. 4). In mice receiving no pravastatin, we observed a 30% higher macrophage content in the plaques of mice immunized with β₂GP1 or with HSA than in non-immunized mice.

**Figure 3:** Cryosections of aortic roots stained by Masson’s Trichrome. Collagen is stained in blue, smooth muscle cells are stained in red. Lipid pool corresponds to the empty area. Scale bar = 200 µm.

**Figure 4:** Collagen content of atherosclerotic lesions in aortic roots. Top: cryosections of aortic roots stained with Sirius red. Scale bar = 500 µm. Bottom: quantification of collagen in aortic roots. Results are normalized to total plaque area and are expressed as percentage. Four mice per group and two sections per mouse were analysed. Values are mean ± SEM. *p<0.05.
Pravastatin treatment resulted in a slightly lower macrophage content that reached significance only in HSA-immunized mice (Fig. 5A). A significantly lower SMC content was observed in the β2GP1-immunized mice than in the HSA-immunized or the non-immunized control mice. Treatment of the β2GP1-immunized mice with pravastatin resulted in SMC contents that were comparable to the HSA-immunized mice (with or without pravastatin) or non-immunized mice (Fig. 5B). CD4 immunohistochemical analysis showed a scattered staining. No significant differences were observed between the groups (Fig. 5C).

Figure 5: Macrophage (A), smooth muscle cell (SMC) (B) and CD4 (C) content in atherosclerotic lesions in aortic roots. Left: Representative panels showing presence of macrophages (A), SMC (B) and CD4 (C) in atherosclerotic plaques of aortic roots. Macrophage staining was done using rabbit anti-mouse Mac 3 antibodies (Scale bar = 500 µm), SMC staining using rat anti-mouse sm-MHC antibodies (Scale bar = 500 µm), and CD4 positive cells using rat anti-mouse CD4 antibodies (Scale bar = 200 µm). Right: Quantitative assessment of macrophage, SMC and CD4 positive cells content. Results are normalized to total plaque area and expressed as percentage of area. Four mice per group and two sections per mouse were analysed. Values are mean ± SEM. *p<0.05, **p<0.01.
Gene expression analysis

The major complication of atherosclerosis is plaque rupture and the ensuing massive activation of the coagulation system, leading to thrombotic occlusions of arteries. To determine whether immunization with β2GP1 or HSA (and statin treatment) has an effect on parameters known to modify plaque stability and coagulation-system activation, we measured mRNA levels of several cell type-specific proteins and inflammation-associated proteins in the thoraco-abdominal aorta. We measured mRNA levels of the following cell-type marker proteins: CD68, a macrophage marker; CD4, a T-lymphocyte marker; and CD25, the interleukin-2 receptor α-chain, a marker of activated T cells. Expression of CD68 was similar in control mice and HSA- or β2GP1-immunized mice without pravastatin, whereas pravastatin treatment resulted in a lower CD68 expression (Fig. 6A). We observed no difference for CD4 (Fig. 6B). Compared to control mice, CD25 expression was 50-fold higher in β2GP1-immunized mice and 20-fold higher in HSA-immunized mice. Pravastatin treatment reduced CD25 expression by 50% in β2GP1-immunized mice (p = 0.10) and had no effect in HSA-immunized mice (Fig. 6C).

mRNA levels of proteins known to modify plaque stability were also measured: interferon-gamma (IFN-γ), reduces plaque stability by decreasing collagen secretion by SMC and by contributing to SMC apoptosis; MMP-9, a protease that is known to destabilize atherosclerotic plaques; TF, the principal trigger of blood coagulation; MCP-1, the monocyte chemoattractant protein; iNOS, the inflammation inducible NO synthase; and IL10, the anti-inflammatory cytokine. We observed that immunization with both β2GP1 and HSA resulted in an increased expression in IFN-γ, MMP-9, TF and MCP-1 and to a lesser extent iNOS (Fig. 7). Consistently, the increase in β2GP1-immunized mice was significantly higher (2- to 3-fold) than in HSA-immunized mice. Pravastatin treatment resulted in a reduction in mRNA levels of most of the marker proteins in the β2GP1-immunized mice, but not in the HSA-immunized mice (Fig. 7). Expression of IL10 was only detectable in the non-immunized group and in two mice in the β2GP1-immunized mice receiving pravastatin (not shown). Interestingly, these two mice IFN-γ expression was very low.

Discussion

The presence of anti-β2GP1 antibodies has been associated with accelerated atherosclerotic lesion development. In the present study, we investigated whether anti-β2GP1 antibodies had an effect on the development and cellular composition of advanced atherosclerotic plaques. We observed that immunization of LDLR-deficient mice with human β2GP1, or HSA as an immunization control, resulted in the formation of autoantibodies against murine β2GP1, or mouse albumin, respectively. After 14 weeks on a high-fat diet, all animals had developed advanced lesions. No difference was noted in lesion size in the thoraco-abdominal aortas or aortic roots of mice immunized with either β2GP1 or HSA, compared to non-immunized mice. A previous study had shown that immunization of LDLR-deficient mice with β2GP1 accelerated the formation of early lesions in chow-fed mice, but had, as in our study, no effect on the size of the

Figure 6: Expression of CD68 (A) CD4 (B) and CD25 (C) in atherosclerotic lesions. Results of real-time quantitative RT-PCR for cell markers within the aorta are shown. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005. The groups of mice analyzed were: Control group n = 8, HSA – pravastatin n = 5, HSA + pravastatin n = 6, β2GP1 – pravastatin n = 5, β2GP1 + pravastatin n = 4.
lesions in mice fed a high-fat diet (13). Plaque size may not be the most relevant clinical parameter. Lethal complications of atherosclerosis, such as unstable angina and myocardial infarction, are associated with plaque rupture, which is more dependent on plaque composition than on plaque size (11). For this reason our study focused on measuring changes in plaque composition and analysis of plaque vulnerability-associated inflammatory marker proteins.

Analysis of the composition of lesions revealed a reduced SMC content and collagen in the β_{2}GP1-immunized mice, but not in the HSA-immunized mice. This may be related to higher expression in the aorta of β_{2}GP1-immunized mice of IFN-γ (an inhibitor of SMC proliferation and collagen synthesis) and of MMP-9 (a collagen degrading proteinase) (11, 12). As SMC and collagen assure fibrous-cap stability, their loss is an important factor leading to plaque weakening and rupture (11). In our analysis, immunization with β_{2}GP1 or HSA increased macrophage content of the root sections, but had no effect on macrophage content of the thoraco-abdominal aorta. This difference might reflect a slower progression of atherosclerotic lesions within the abdominal aorta (22). An analysis of CD4 content of the plaques did not reveal any differences between control mice and β_{2}GP1- or HSA-immunized mice.

An important aspect of atherosclerosis, in addition to plaque composition, is the activation state of cells within the plaques. To analyse this, we measured mRNA levels of several inflammation-associated proteins. Our results revealed that immunization with β_{2}GP1 or HSA resulted in markedly higher expression of CD25, a marker of activated T cells in the atherosclerotic lesions. Moreover, we measured increased expression of inflammation-associated proteins such as IFN-γ, MMP-9, TF, MCP-1 and iNOS. Increases were greater in the β_{2}GP1-immunized mice than in the HSA-immunized mice. Our data therefore suggest that the presence of autoantibodies, irrespective whether these are directed against β_{2}GP1 or albumin, is a major factor responsible for a more inflammatory phenotype of advanced lesions. We also measured mRNA levels of IL-10, an important anti-inflammatory cytokine. It was detectable in all control mice and undetectable in most of the immunized mice. These data suggest that immunization with β_{2}GP1 and HSA reduces IL-10 expression.

Taken together, our data imply that immunization with β_{2}GP1 or HSA increases the inflammatory phenotype of atherosclerotic plaques. The alterations appear to be greater in β_{2}GP1-immunized mice than in HSA-immunized animals. Our data do not allow us to determine whether the phenotypic differences between β_{2}GP1- and HSA-immunized mice are due to spe-

![Image](https://example.com/image.png)

**Figure 7:** Expression of inflammatory markers within lesions. Results of real-time quantitative RT-PCR for pro-inflammatory molecules within the aorta are shown: IFN-γ (A), MMP-9 (B), TF (C), MCP-1 (D), and iNOS (E). Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005. The groups of mice analysed were: Control group n = 8, HSA – pravastatin n = 5, HSA + pravastatin n = 6, β_{2}GP1 – pravastatin n = 5, β_{2}GP1 + pravastatin n = 4.
cific anti-β₂GP1-mediated effects, to different autoantibody levels or to a different stoichiometry between antibodies and antigen. Plasma concentrations of albumin are much higher than those of β₂GP1 and therefore even with similar antibody concentrations, the nature of immune complexes is likely to be different. The observation that pravastatin treatment reduced the response to immunization with β₂GP1 but not with HSA suggests that immunization with β₂GP1 has an additional pathogenic effect. One mechanism may be specifically related to β₂GP1. Anti-β₂GP1 antibodies and aPLA are known to activate endothelial cells and monocytes (9, 10, 23). Therefore, these antibodies may facilitate the influx and activation of monocytes into lesion areas. In addition, anti-β₂GP1 antibodies may cross-react with oxidized LDL, which may then act as an additional pathogenic factor (24). Alternatively, immunization with β₂GP1 or HSA may have led to the formation of circulating immune complexes with murine β₂GP1 or albumin. The presence of such complexes induces a persistent inflammatory state that may accelerate the development of atherosclerotic lesions and reduce plaque stability (25). In addition, circulating immune complexes activate complement, which is known to contribute to atherosclerosis (26) and also to the pathological effects of aPLA (27). Finally, the process of immunization itself, in particular the adjuvant used, may contribute to atherosclerosis. Freund’s complete adjuvant accelerated atherosclerosis development in rabbits, whereas RIBI™, the adjuvant used in our present study, had no effect (28). In rabbits fed a cholesterol-rich diet, BCG immunization was associated with increased surface expression of CD25 on peripheral lymphocytes, increased monocyte recruitment and enhanced atherosclerosis compared with non-immunized controls (29). These studies imply that in atherosclerotic lesions, immune responses increase the expression of inflammatory proteins known to contribute to plaque weakening and rupture.

Statins are known to reduce the risk of atherosclerotic complications (11, 17, 30). This may be due to a reduction of cholesterol levels, but also to pleiotropic anti-inflammatory effects. To establish whether treatment with statins reduces the severity of the phenotypic changes induced by immunization, we also included animals receiving pravastatin. We observed, as expected, that statin treatment reduced lesion development in both β₂GP1- and HSA-immunized mice. This effect was most pronounced in the thoraco-abdominal aorta, but also evident in the aortic roots. Statin treatment had little effect on collagen or macrophage content in the lesion areas. However, in β₂GP1-immunized mice, statin treatment increased SMC content and reduced the expression of CD68, CD25, and TF and several inflammation-associated proteins. The effect of statins on plaque composition has been studied by several groups. In APOE*3-Leiden transgenic mice, atorvastatin had no effect on the collagen content of existing atherosclerotic plaques (31). In contrast, we previously observed a thicker fibrous cap and reduced number of inflammatory cells in LDLR-deficient mice receiving a high-cholesterol diet and pravastatin in their drinking water (32). The effect of statin treatment on metalloproteinase activity is well established. In cultured macrophages, MMP-9 synthesis and activity are decreased by fluvastatin (33). In Watanabe heritable hyperlipemic rabbits, receiving pravastatin or fluvastatin, expression of MMP-1, MMP-3 and MMP-9 by macrophages in the atheroma was lower than in a control group, whereas there was no difference in macrophage numbers (34).

Taken together, our results show that the presence of antibodies to β₂GP1 leads to an inflammatory phenotype in atherosclerotic lesions, which may contribute to plaque weakening and ultimately plaque rupture. However, most of the effects do not appear to be specific for β₂GP1, because immunization with HSA led to similar, albeit less severe, phenotypic changes. Immunization of LDLR-deficient mice with β₂GP1 or albumin may be a useful approach to create in-vivo models to study mechanisms leading to plaque weakening.

References
1. Bruce IN. ’Not only...but also’: factors that contribute to accelerated atherosclerosis and premature coronary heart disease in systemic lupus erythematosus. Rheumatology 2005; 44: 1492–502.