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# Suppression of early atherosclerosis in LDL-receptor deficient mice by oral tolerance with β2-glycoprotein I

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**Abstract**

**Background:** Atherosclerosis is considered analogous to chronic inflammatory diseases. Beta2-glycoprotein I (β2GPI) is a phospholipid binding protein shown to serve as a target for prothrombotic antiphospholipid antibodies. It has recently been demonstrated to drive an immune mediated reaction and enhance murine atherosclerosis. Oral tolerance is a method in which feeding a given antigen, downregulates the respective immune responses towards it, and attenuates concomitant organ specific disorders. Herein, we tested the hypothesis, that inhibiting cellular immunity to β2GPI would result in suppression of fatty streak formation in mice. **Methods and results:** LDL receptor deficient mice were fed different doses of human or bovine β2GPI or BSA and than switched to an atherogenic diet. To determine the effect of feeding on lymph node proliferative indices, separate groups of mice were fed β2GPI and then immunized with the respective antigen. Feeding either human or bovine β2GPI was effective in attenuating atherosclerosis as compared to control fed animals. Oral feeding with of β2GPI inhibited lymph node cell reactivity to β2GPI in mice immunized against the human protein. Oral tolerance was also capable of reducing reactivity to oxidized LDL in mice immunized against oxLDL. IL-4 and IL-10 production was upregulated in lymph node cells of β2GPI-tolerant mice immunized against β2GPI, upon priming with the respective protein. **Conclusion:** Thus, oral administration of β2GPI is an effective means of suppressing atherogenesis in mice and should further be investigated.


*Keywords:* Atherosclerosis; Oral tolerance; Immunology; Lymphocyte; β2GPI

## 1. Introduction

Atherosclerosis is a multifactorial histological process that involves uninhibited lipid accumulation within the vessel wall [1]. In recent years, considerable data has accumulated to support a dominant role for innate and adaptive immune responses in atherogenesis [2]. These observations have led to the contention that atherosclerosis is analogous to a chronic inflammatory process. The idea that immune effectors and soluble mediators prevail within the atherosclerotic plaque, had been further extended recently, to suggest that modified antigens or cross-reactive responses are involved in determining the size and composition of the lesion [3]. In this context, several autoantigens have been shown to evoke respective immune response and have thus been considered as target of an autoimmune-like response occurring locally. This paradigm although apparently provocative could pave the way for a new line of treatment strategies based on antigen-specific modulation, thus avoiding the hazardous implications of general immunosuppression.

Prototypic antigens incriminated in atherosclerosis are modified forms of lipoproteins [4], heat shock protein 60/65 [5] and other antigenic molecules within infectious agents

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(reviewed in Ref. [3]). In a set of recent studies we have shown that β2-glycoprotein I (β2GPI), a phospholipid binding protein [6], is expressed within atherosclerotic lesions and induces an immune response that enhances fatty streak formation in mice [7–9]. β2GPI is a circulating glycoprotein that has been shown to serve as a target of anti-phospholipid antibodies in patients with a procoagulant state and with associated autoimmune disorders (predominantly SLE) [10]. However, β2GPI has also been shown to bind apoptotic cells thus possibly serving as a transporter and regulator of cellular trafficking [11].

Mucosal tolerance is a method by which prior exposure of a given antigen induces a state of selective unresponsiveness with a resultant attenuation of the disorder [12,13]. Several experimental animal models have been tested with regard to mucosal tolerance and include: experimental autoimmune encephalomyelitis (EAE), diabetes mellitus and rheumatoid arthritis models. However, it appears that not only immunologically induced experimental models can improve following mucosal tolerance but also insults such as stroke in animals [14,15].

We have recently shown that β2GPI, administered orally, was capable of suppressing a murine syndrome that mimics the prothrombotic antiphospholipid syndrome [16]. In the current study, we evaluated the efficacy of β2GPI, a proposed candidate antigen in atherogenesis, as a tolerating therapeutic agent in atherosclerosis employing oral tolerance.

## 2. Materials and methods

### 2.1. Mice

LDL-receptor deficient (RD) mice [17] were females at the age 6 weeks, obtained from the Jackson Laboratories and bred at the local animal house. The mice were either fed normal chow-diet or an atherogenic diet (Western type diet, TD 96125, Harlan Teklad; 42% of calories from fat, 43% from carbohydrates, 15% from protein; 0.15% by weight cholesterol).

### 2.2. Preparation of β2GPI

Human and bovine β2GPI were prepared from pooled plasma as described previously [8]. To ensure purity, pooled plasma humans or mice was subsequently chromatographed on a heparin-Sepharose column, on a DEAE-cellulose column, and on an anti-β2GPI affinity column. To remove any contamination by IgGs, the β2GPI-rich fraction was further passed through a protein A-Sepharose column.

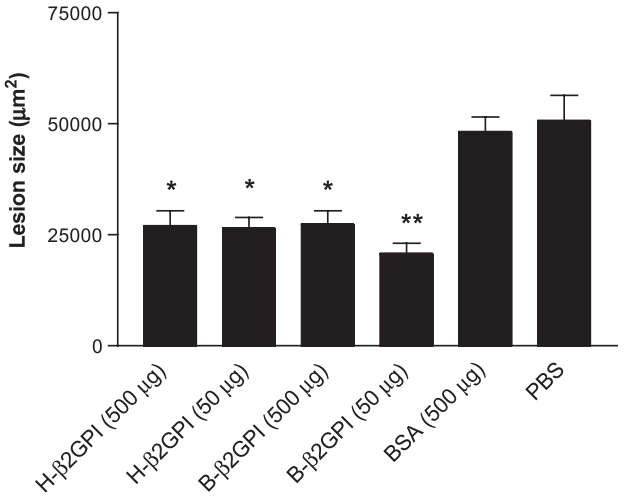


Fig. 1. Oral tolerance with β2GPI attenuates atherosclerosis in LDL-RD mice. Human (H-β2GPI) and bovine (B-β2GPI) β2GPI, BSA or PBS were fed to LDL-receptor deficient mice (16–17 mice per group) and a Western diet was then commenced for 4 weeks. Atherosclerotic lesions were determined at the aortic sinus. *$p < 0.001$ as compared with BSA fed; **$p < 0.0001$ as compared with BSA fed.

### 2.3. Cholesterol level determinations

Total plasma cholesterol levels were determined by using an automated enzymatic technique (Kit No. 816302; Boehringer, Mannheim, Germany).

### 2.4. Study design

LDL-RD mice were fed by a nasogastric tube, five doses (every other day) of human or bovine β2GPI in PBS in two different concentrations (500 and 50 μg/dose). Control mice were either fed an irrelevant antigen (BSA; 50 μg) or not-fed. One day following the last feeding, all mice were switched from chow-diet to Western diet and sacrificed 4 weeks later. Study was approved by the local Ethics committee in accord with the NIH guidelines.

Table 1
Lipid profile in weight in mice tolerated orally with β2GPI

| | | H-$\beta_2$ GPI (500 μg) | H-$\beta_2$ GPI (50 μg) | B-$\beta_2$ GPI (500 μg) | B-$\beta_2$ GPI (50 μg) | BSA (500 μg) | PBS |
|---|---|---|---|---|---|---|---|
| Start | Weight (g) | 27.1 ± 0.7 | 26.4 ± 0.7 | 25.7 ± 0.7 | 26.4 ± 0.7 | 26.8 ± 0.7 | 26.5 ± 0.6 |
| | Chol (mg/dl) | 231 ± 16 | 227 ± 14 | 233 ± 15 | 231 ± 14 | 231 ± 17 | 232 ± 12 |
| | Tg (mg/dl) | 182 ± 18 | 212 ± 26 | 178 ± 23 | 179 ± 20 | 188 ± 26 | 189 ± 23 |
| End | Weight (g) | 31.4 ± 1.0 | 32.0 ± 0.6 | 29.8 ± 0.9 | 30.8 ± 0.8 | 30.5 ± 0.8 | 31.6 ± 0.6 |
| | Chol (mg/dl) | 1232 ± 92 | 1237 ± 71 | 1196 ± 75 | 1200 ± 76 | 1166 ± 91 | 1250 ± 90 |
| | Tg (mg/dl) | 415 ± 62 | 342 ± 51 | 318 ± 55 | 313 ± 51 | 293 ± 45 | 424 ± 54 |

H-β2—human β2GPI.
B-β2—bovine β2GPI.

### 2.5. Proliferation assays

A separate group of mice were fed with bovine β2GPI or BSA and immunized with β2GPI, 1 day following the last fed dose. Draining inguinal lymph nodes (taken 8 days after immunization were collected from three mice from each of the groups, for the proliferation studies. The assays were performed as previously described [8] with minor modifications. Briefly, $1 \times 10^6$ cells per ml were incubated in triplicates for 72 h in 0.2 ml of culture medium in microtiter wells in the presence 10 mg/ml β2GPI, or BSA. Proliferation was measured by the incorporation of [$^3$H] thymidine into DNA during the final 12 h of incubation. The results were computed as stimulation index (S.I.): the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen. Standard deviation were always $<10\%$ of the mean cpm.

An additional experiment was conducted to assess the effect of oral tolerance with β2GPI on reactivity to oxLDL. For this purpose, mice ($n=4$) were either tolerated with β2GPI or BSA for five doses. One day following the last dose, all mice were immunized with human oxLDL or BSA (10 μg/ml) and draining lymph nodes were collected 10 days later. Proliferation to oxLDL was assessed by thymidine incorporation following a 72-h incubation with oxLDL.

### 2.6. IFN-γ, IL-4, IL-10 and TGF-β secretion by tolerated lymph node cells

Conditioned medium was obtained from the lymph node proliferation experiments following 48 h of culture in the presence of β2GPI. IFN-γ, IL-4, IL-10 and TGF-β concentrations were determined by ELISA kits according to the manufacturer's suggestions (R&D systems).

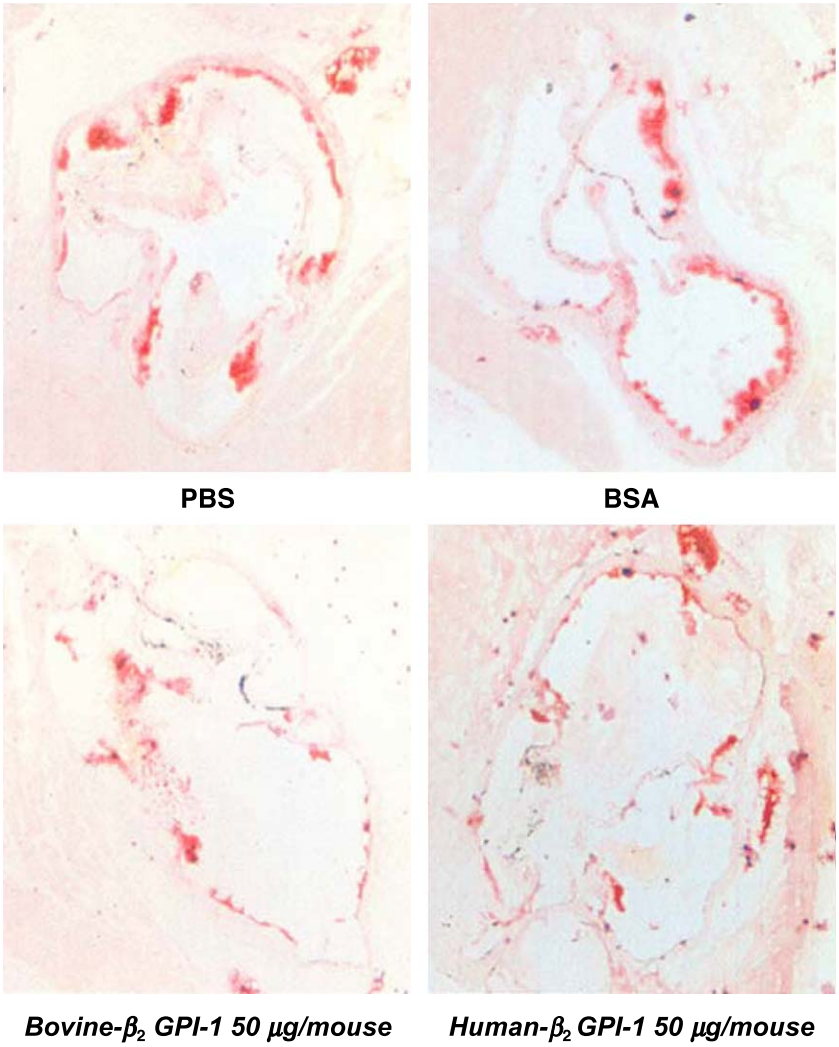


Fig. 2. Representative oil-red O stained lesions from β2GPI-tolerant mice. Upon sacrifice, hearts were removed from all mice and assessed for atherosclerosis following staining with oil-red O.

### 2.7. Detection of anti-β2GPI antibodies and antibody isotypes

β2GPI (10 μg/ml) was coated onto flat bottom 96-well ELISA plates (Nunc) by overnight incubation and the assay was performed as previously described [8].

IgG, IgA and IgM isotypes in the sera of β2GPI tolerant and non-tolerant mice were determined by an ELISA kit (Southern Biotechnology Associates, Birmingham, AL, USA) according to the manufacture's instructions.

### 2.8. Assessment of atherosclerosis

Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described [8]. with a few modifications. Briefly, the heart and upper section of the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section was embedded in OCT medium and frozen. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. The extent of atherosclerosis was evaluated at the level of the aortic sinus (expressed as mean area per aortic sinus). Processing and staining of the tissue with oil-red O was carried out according to Paigen et al. [18].

### 2.9. Immunohistochemistry of atherosclerotic lesions

Immunohistochemical staining for CD3, macrophages and β2GPI content were done on aortic sinus 5-μm-thick frozen sections. Primary antibodies used for probing were rat anti-mouse CD3, rat anti-mouse Mac-1 and a polyclonal rat anti-mouse β2GPI antibodies [9]. Slides were developed with the three amino-9-ethylcarbonasole (AEC) substrate. Sections were counterstained with hematoxylin. Spleen sections were used as a positive control. Staining in the absence of 1st or 2nd antibody was used as a negative control. β2GPI presence were evaluated by its occupancy of plaque area by computerized morphometry as described previously for VCAM-1 [19].

### 2.10. Statistical analysis

All parameters were compared employing a one-way ANOVA test. $P < 0.05$ was accepted as statistically significant. Results are presented as mean ± S.E.M.

## 3. Results

Oral feeding of human or β2GPI did not significantly influence total cholesterol or triglyceride levels (Table 1).

Feeding with mice with BSA did not alter lesion progression in comparison with PBS. Oral administration of human β2GPI at 50 μg and at 500 μg/dose were similarly effective in suppressing atherosclerosis in the mice (45% and 44% reduction, respectively, as compared with BSA-fed). Oral tolerance with bovine β2GPI was also effective in reducing early atherosclerotic size in both 50 μg and at 500 μg dosages (57% and 43% suppression, respectively) (Figs. 1 and 2).

Oral tolerance with β2GPI (500 μg/dose) did not influence the content of plaque expressed β2GPI (mean percent occupancy of 15 ± 5) in comparison with BSA feeding (occupancy of 19 ± 7%). Oral tolerance did not influence the inflammatory phenotype of the fatty streaks evident by the lack of effect on the number of CD3 positive cells (0–5 cells/plaque in all groups; data not shown) or macrophage (Mac-1 positive) content.

In order to investigate whether β2GPI administration was associated with tolerance to the respective protein, in vivo,

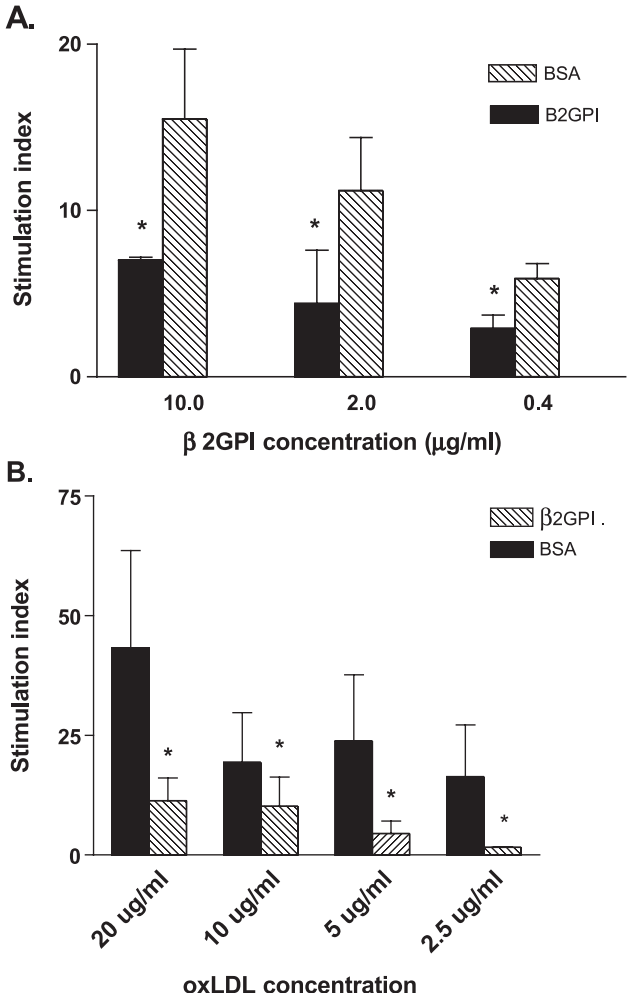


Fig. 3. Oral feeding with β2GPI inhibits cellular immune responses to β2GPI and oxLDL. Lymph-node cells from bovine β2GPI or BSA fed, β2GPI immunized, were assessed for thymidine uptake in the presence of different concentrations of β2GPI (A). Lymph node cells were collected from mice that were tolerant with β2GPI or BSA and immunized against oxLDL. Reactivity to oxLDL was determined by thymidine incorporation following incubation with oxLDL (B). *$p < 0.05$.

we have performed the lymph-node proliferation assays. Oral feeding of bovine β2GPI, at the dosage successfully employed to suppress atherosclerosis (Fig. 3A) significantly inhibited lymph-node proliferation to human β2GPI in immunized mice.

Next, we went on to study the effect of oral feeding with β2GPI on the primary cellular immune responses to oxLDL. We have found that prior β2GPI feeding (at five doses of 500 μg/mouse) significantly reduced lymph node reactivity to oxLDL as compared with BSA feeding (74% suppression upon stimulation with 20 μg/ml of oxLDL) (Fig. 3B). Feeding of β2GPI did not influence stimulation indices to BSA in mice immunized with BSA; Moreover, BSA feeding did not reduce the proliferative response to oxLDL in mice immunized with oxLDL (data not shown).

To investigate whether suppression of lymph node cell reactivity to β2GPI was associated with a skewed cytokine production, conditioned medium was collected from β2GPI-tolerant and non-tolerant cells from mice immunized with β2GPI (50 μg/mouse) after 48 h of incubation in the presence of β2GPI. Levels of IL-4 in medium from β2GPI-tolerant cells were three times higher ($p < 0.01$) as compared with non-tolerant lymph node cells (Fig. 4). A similar pattern was evident with regard to IL-10. Namely, lymph node cells from β2GPI-tolerant mice immunized with β2GPI secreted significantly higher IL-10 in comparison with non-tolerant cells (2.6 times higher; $p < 0.05$) upon in vitro priming with β2GPI (Fig. 4).

β2GPI feeding did not induce significant changes in the levels of secreted IFN-γ (mean value of 1802 ± 588 pg/ml in the β2GPI-tolerant cells as compared with 1870 ± 378 pg/ml in the non-tolerant). TGF-β levels in the conditioned medium of lymph node cells obtained from tolerant and non-tolerant mice were below the detection threshold.

To explore whether Th2 cytokine dominance was associated with a skewed antibody isotype distribution, we evaluated their total levels as well as the anti-β2GPI IgM, IgG and IgA antibody levels and isotypes in sera of tolerant mice that were subsequently immunized with β2GPI. Oral tolerance with β2GPI did not alter IgM, IgA, or IgG total antibody levels nor was there a change in isotype distribution in comparison with non-tolerant mice (data not shown).

None of the fed antigens induced production of anti-β2GPI antibodies (data not shown).

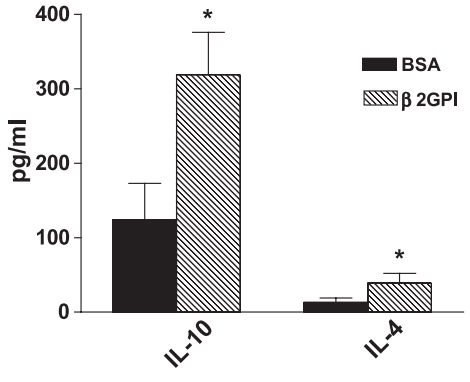


Fig. 4. Oral tolerance with β2GPI induces Th2 cytokine production by β2GPI reactive cells. Conditioned medium was collected from lymph node cells of mice orally tolerated with β2GPI or BSA, immunized with β2GPI and incubated with β2GPI (10 μg/ml) for 48 h. Levels of IL-4 and IL-10 were probed in the medium employing a capture ELISA kit as described in Materials and methods. *$p < 0.01$.

## 4. Discussion

In the current study, we have shown that oral tolerance with human and bovine β2GPI was capable of suppressing early atherosclerotic lesions in LDL-receptor deficient mice.

The paradigm of autoimmune like reactions generated towards plaque expressed neo- or cross-reactive antigens is relatively novel [3]. In this context, the most extensively studied antigens are modified lipoproteins (in particular, oxLDL) and heat shock proteins. Autoimmunity to β2GPI has mostly been investigated in SLE patients and in subjects with the prothrombotic primary antiphospholipid syndrome. Antibodies to β2GPI have been suggested to provoke the procoagulant state due to multiple effects on platelets, endothelial cells and plasma coagulation products (reviewed in Ref. [20]). Recent evidence suggests that cellular immunity to β2GPI is also operable and can induce cytokine shift in lymphocytes from antiphospholipid syndrome patients [21].

We have recently provided evidence to support the involvement of β2GPI in atherosclerosis in a set of studies. Immunization of mice with β2GPI induced early fatty streaks that were transferable by lymph node and spleen cells from the primed animals [8,9]. Moreover, β2GPI was also expressed in human plaques and colocalized with CD4 lymphocytes [7]. We have also obtained evidence that β2GPI is expressed in atherosclerotic plaques of LDL-receptor-deficient mice [9]. It has thus been proposed that cellular and humoral immunity to β2GPI could potentially contribute to fatty streak formation.

The application of oral tolerance as a therapeutic strategy has proven successful in various immune and non-immune mediated experimental models, yet efficacy in human disease is still pending [12,13]. Several mechanisms have been provided to explain the beneficial effects of oral tolerance. The two major mechanisms suggested are the induction of clonal anergy or deletion by high doses of fed antigen [22,23], versus active cellular regulation [24,25] brought about by lower antigen dose feeding. In the current study, we observed that prior feeding with β2GPI was capable of significantly inhibiting the reactivity of lymph node cells from β2GPI to the respective antigen. Thus, clonal deletion/anergy could have played a role in protection, as lymphocytes reactive with β2GPI were recently shown by us as directly responsible for accelerating atherogenesis [9]. The significance of clonal deletion in this model is somewhat more questionable due to the tolerating effects obtained in

lower doses that is more appropriately explained by active suppression, although it could be that different mechanisms of protection were operable in the low and high doses.

It should be mentioned that evidence for the role of β2GPI-autoimmunity in non-manipulated animals is not available and the specificity of this mechanism should thus be interpreted with caution. The lack of spontaneous reactivity to β2GPI may be due to relatively young age of the animals that has not yet developed even with high levels of anti-oxLDL antibodies. Relevant to this suggestion is the finding by Pratico et al. [26] of the presence of antibodies to oxidized phospholipids in older apoE KO (26 weeks), whereas in our studies transgenic mice were 10 weeks old at sacrifice. An additional explanation may be related to the inappropriate presentation of cryptic 'autoimmune' epitopes on β2GPI on the solid phase assay, which could unmask autoimmune-like reactions both in solid (ELISA) and fluid phase (proliferation) assays.

Several autoantigens have been proposed to trigger autoimmune like reaction with the plaque. The most commonly investigated is oxidized LDL which have been shown to be present abundantly within human and animal atheroma (reviewed in Ref. [4]). Recent observations by Nicoletti et al. [27] imply that autoimmunity to oxLDL may be proatherogenic as tolerance to the antigen, brought about by neonatal injection, led to clonal anergy/deletion of oxLDL reactive cells and to consequent suppression of atherosclerosis. We have thus hypothesized that prior feeding with β2GPI may also influence reactivity to oxLDL in a bystander manner. Bystander suppression relates to the ability of a given antigen to confer tolerance to other non-related antigen [28]. This phenomenon has been demonstrated previously in experimental allergic encephalomyelitis and adjuvant arthritis (reviewed in Refs. [12,13]). Indeed, we have found that β2GPI fed prior to oxLDL immunization could significantly inhibit primary immune responses to oxLDL, similar to the suppressive effect it induced on β2GPI reactivity, thus providing an additional explanation for the reduced plaque formation.

We have also observed that toleration of immunity to β2GPI by feeding the respective protein was associated with production of significantly higher levels of the T-helper cytokines IL-4 and IL-10 upon in vitro priming with β2GPI. As our previous [9] as well as current data show that β2GPI is present abundantly with atheroma, it is possible that ligation of T cell receptor by the 'educated' lymphocytes (established following β2GPI toleration) results in Th2 cytokine induction. IL-4 and IL-10 have been demonstrated to play an instrumental role in conferring protection following oral tolerance [12,13]. Studies in mice deficient in both these cytokines are particularly suggestive in this context [29]. The relevance to atherosclerosis can be understood in view of the work of Mallat et al. [30] demonstrating that IL-10 is an anti-atherogenic cytokine in mice. However, in the present study, Th1 dominance upon antigen-specific stimulation was insufficient to inhibit Th1 response, as levels of IFN-γ in conditioned medium from β2GPI tolerant and non-tolerant mice did not differ significantly. Several anti-atherogenic properties of IL-10 are possible irrespective of Th1 cytokine suppression: inhibition of the prototypic pro-inflammatory transcription factor nuclear factor-B, inhibition of matrix metalloproteinases, reduction of tissue factor expression, and inhibition of apoptosis of macrophages and monocytes.

Interestingly, TGF-β production did not increase as a result of antigen feeding, as observed in several previous studies. TGF-β levels were found to be reduced in atherosclerosis [31]. The reduction of TGF-β associated with atherosclerosis, may have thus suppressed cytokine levels to a degree that can not be counter regulated by β2GPI feeding.

An important issue is the similar effects provided by both doses (50 and 500 μg) on atherosclerosis. It is possible that both doses are above the values needed to confer tolerance. Moreover, both human and bovine β2GPI were effective in suppressing atherosclerosis suggesting both may harbor the active site needed to provide the tolerating effect.

We did not observe a change in inflammatory cell infiltration or macrophage content in orally tolerant mice as compared with controls. This finding may be related to a change in cytokine secreting phenotype of inflammatory cells rather than to their capacity to infiltrate the lesions resulting in similar plaque containing cells with a predominantly skewed Th2 production. As different factors control fatty streak and complex lesion development, it is warranted to carry out similar studies in more advanced atherosclerotic plaques.

In conclusion, we have demonstrated for the first time, the feasibility of oral tolerance with β2GPI, in attenuating fatty streak formation in mice concomitant with suppression of the cellular immune response to the respective protein. This strategy should further be investigated in animal models of atherosclerosis (including in males) before firm conclusions can be drawn.

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