Subclinical Atherosclerosis in Systemic Lupus Erythematosus and Antiphospholipid Syndrome
Focus on β2GPI-Specific T Cell Response

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Objective—Systemic Lupus Erythematosus (SLE) and antiphospholipid syndrome (APS) are associated with a high prevalence of atherosclerosis. β2 glycoprotein I (β2GPI) represents a link between autoimmunity and endothelial dysfunction. Recently, β2GPI reactive T cells have been identified; however, their role in atherosclerosis is still under investigation. We evaluated early atherosclerosis in patients with SLE and APS and investigated T cell reactivity to β2GPI and its relationship with atherosclerotic process.

Approach and Results—Fifty SLE, 18 patients with primary APS (PAPS), and 25 healthy controls were enrolled. Demographic and clinical data, including traditional cardiovascular risk factors, were recorded. Monocyte β2GPI and Tissue Factor (TF) expression and peripheral blood mononuclear cell response to β2GPI stimulation were evaluated. Doppler ultrasound was performed to investigate flow-mediated dilatation (FMD) and carotid intima-media thickness (IMT). We detected an increase in mean IMT and a decrease in FMD in patients with SLE versus controls (P<0.05 and P=0.001, respectively) and a decrease in FMD in patients with PAPS versus controls (P<0.05). Monocyte β2GPI and TF expression was higher in patients with SLE and PAPS than in controls (P=0.006 and P=0.001, respectively); no correlation of monocyte β2GPI and TF with IMT or FMD was detected. β2GPI induced peripheral blood mononuclear cell proliferation in 32% of patients with SLE, 25% of patients with PAPS yet in none of the controls. Proliferative response to β2GPI correlated with a history of arterial thrombosis, thrombocytopenia, and IMT >0.9 mm.

Conclusions—A significant percentage of patients with SLE and PAPS show a β2GPI-specific T cell reactivity, which is associated with subclinical atherosclerosis. (Arterioscler Thromb Vasc Biol. 2014;34:661-668.)

Key Words: antiphospholipid syndrome ■ atherosclerosis ■ beta 2-glycoprotein I ■ systemic lupus erythematosus

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease with a multifactorial pathogenesis including genetic and environmental factors.12 The disease is characterized by the production of a wide range of autoantibodies.3-7 SLE mainly affects women in their reproductive age, and any organ and system can be involved in the pathological process.1,8,9 Moreover, it is well known that patients with SLE present a higher prevalence of early atherosclerosis and have a greater risk of developing cardiovascular diseases than healthy subjects.10,11 Increasing evidence suggests that early atherosclerosis in SLE is associated with both classical cardiovascular risk factors (hypertension, hypercholesterolemia, hypertriglyceridemia, diabetes mellitus) and disease-related risk factors.12 Endothelial dysfunction represents the earliest stage of atherosclerosis, and the change in brachial artery flow-mediated dilatation (FMD) is a well known marker of endothelial dysfunction.13,14 FMD has been consistently reported to be abnormal in subjects affected by atherosclerosis or presenting cardiovascular risk factors, as well as in patients with SLE.15-17 Measurement of the intima-media thickness (IMT) of carotid arteries by Doppler ultrasound also represents a quantitative method for detecting preclinical atherosclerosis. Increased IMT has been observed in patients with SLE with a prevalence of focal plaque up to 37% in patients with SLE without previous cardiovascular events.18-20

In addition, the prevalence of atherosclerosis seems to be increased among patients with antiphospholipid syndrome (APS), an autoimmune disease characterized by arterial and venous thrombosis and obstetric manifestations associated with antibodies against phospholipid-protein complexes.21-26 Of note, the prevalence of carotid plaque has been reported to

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be higher in patients with APS secondary to SLE compared with patients with primary APS (PAPS).27 Moreover, in patients with PAPS, the titer of antiphospholipid antibodies (aPL) often represents an independent predictor of IMT.28,29 This may be consistent with the observation that anticardiolipin antibodies (aCL), mostly β2 glycoprotein I (β2GPI)-dependent aCL, and antiβ2GPI antibodies (aβ2GPI) are important predictors for cardiovascular events.30,31 β2GPI, or apolipoprotein H, is an abundant plasma glycoprotein that binds to negatively charged phospholipids and is involved in clotting mechanisms and lipid pathways.21,32,33 In chronic diseases related to endothelial cell dysfunction such as SLE, APS, and atherosclerosis, β2GPI plays a role as a target antigen for an immune-mediated attack, possibly influencing the progression of disease.24,34–39 β2GPI stimulates not only a vigorous adaptive humoral but also a cellular, immune response.40–43 Recently, the presence of autoreactive T cells specific for a cryptic epitope of β2GPI has been described in both patients with APS and healthy subjects.44–46 Moreover, the frequency of these autoreactive cells was found to be significantly increased in patients with APS, suggesting that the production of aPL in patients with APS could be a result of the activation of β2GPI-reactive T cells. Recently, we showed that β2GPI is a T cell target in patients with advanced carotid atherosclerotic plaques.47 However, the association of β2GPI-reactive cells with the occurrence of atherosclerosis in patients with APS and SLE still needs to be further investigated.

The aim of this study was to evaluate the specific T cell reactivity against β2GPI in patients with SLE and PAPS and the possible association of this reactivity with markers of preclinical atherosclerosis such as endothelial dysfunction and carotid wall thickness.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Clinical Assessment
The demographic and clinical features of patients and controls are shown in Table 1. Although not statistically significant, control subjects were slightly older than both patients with SLE and PAPS, and the disease duration was longer in patients with SLE than in those with PAPS. Cumulative glucocorticoid dosage was significantly higher in patients with SLE than in those with PAPS (P<0.0001). Overall, patients with PAPS showed higher serum levels of aPL than patients with SLE and normal controls (see Table 1 for details).

Table 2 shows the distribution of traditional cardiovascular risk factors in the study population. Body mass index and systolic blood pressure were significantly higher in patients with SLE and PAPS than in controls. Diastolic blood pressure was higher in patients with SLE than in controls. Plasma levels of high-density lipoprotein (HDL)-cholesterol and Apo A-1 were significantly lower in patients with SLE and PAPS compared with controls.

Peripheral Blood Mononuclear Cell Response to β2GPI
β2GPI induced a proliferative response in peripheral blood mononuclear cell (PBMC) samples from 12 of 37 patients with SLE (32%, stimulation index [SI] ranging from 2.2–5.2) and from 3 of 12 patients with PAPS (25%, SI ranging from 3.2–4.2). No samples from healthy controls (n=23) responded to β2GPI (SLE versus controls P=0.002, PAPS versus controls P=0.034). The median SI was higher in samples from patients with SLE and PAPS than in those from healthy controls although the difference was not statistically significant (Figure 1). No positive reactions were observed in cultures stimulated with human serum albumin (data not shown). No difference in T cell proliferation response was observed between patients who were taking immunosuppressants and those who were not. PBMC samples proliferating in response to β2GPI produced higher Interferon (IFN)–γ concentrations than nonproliferating samples from patients (P=0.04) and controls (Table 3 and Figure 1 in the online-only Data Supplement). In contrast, no differences were observed in interleukin-4 production.

The univariate analysis demonstrated a positive correlation between SI values and IFN-γ levels (r=0.3, P=0.04) and between SI values and aβ2GPI IgM (r=0.4, P=0.0004), or aCL IgM levels (r=0.3, P<0.001) in all the enrolled subjects. The proliferative response in PBMC samples was associated with a history of arterial thrombosis and a history of thrombocytopenia and mIMT ≥0.9 (P=0.035 for the 3 parameters). In addition, a proliferative response in PBMC samples was associated with a history of anti-nuclear antibodies and anti-dsDNA serum positivity (P<0.005). No correlation was found between a proliferative response in PBMC samples and FMD.

β2GPI and TF Expression on Human Monocytes
Cytofluorimetric analysis with aβ2GPI showed a significantly higher staining for β2GPI on monocytes (% reactive cells±SD) from patients with SLE (34.2±18.9) and PAPS (36.3±22.7) compared with those from healthy controls (18.6±13.7; P=0.006 and P=0.001, respectively). Dual staining for β2GPI and TF showed a significantly higher staining on monocytes from patients with SLE (69.5±20.6) and PAPS (66.9±26.9) than from healthy controls (18.6±13.7; P=0.00001 and P=0.001, respectively). One representative example of β2GPI and TF staining on monocytes from a patient with PAPS versus a healthy control is shown in Figure 2.
IMT and FMD Correlations With Traditional and Disease-Related Risk Factors for Atherosclerosis

Table 4 summarizes the ultrasonographic findings in patients and controls. Compared with controls, mean carotid IMT was higher in both patients with SLE and PAPS, although in PAPS the difference was not statistically significant probably because of the limited number of enrolled patients. In one 69-year-old female patient with SLE and secondary APS, an atherosclerotic plaque was detected. FMD was significantly impaired in patients with SLE (expressed both as an absolute value and as a percentage of increase from basal value) when compared with the other 2 study groups (see Table 4 for details). Nineteen patients with SLE had secondary APS (mean age comparable with that of patients with PAPS): these patients did not show a significant change in mean carotid IMT but presented a significantly reduced FMD (both absolute value and percentage of increase from basal value) when compared with patients with PAPS (Table 5).

Univariate analysis showed that mean carotid IMT in patients with SLE was associated with systemic lupus international collaborative clinics/American College of Rheumatology damage index (SDI) \( (P=0.001) \). As reported in Table 6, stepwise multivariate analysis confirmed SDI as an independent variable correlated with mean carotid IMT in patients with SLE. Concerning FMD (%), univariate analysis showed an inverse correlation with plasma cholesterol levels in patients with SLE \( (P=0.01) \) that was confirmed by step-wise multivariate analysis (Table 4). In addition, stepwise multivariate analysis showed a correlation between FMD (%) and cumulative glucocorticoid dosage (Table 4).
Univariate analysis in patients with PAPS demonstrated that mean carotid IMT was related to age (P<0.0001), disease duration (P=0.008), systolic blood pressure (P<0.0001), diastolic blood pressure (P=0.016), plasma cholesterol levels (P=0.008), plasma triglycerides levels (P=0.001), aCL IgM (P<0.0001), and aβ2GPI IgM (P<0.0001). Only age and aβ2GPI IgM were confirmed as independent variables associated with mean carotid IMT once stepwise multivariate analysis was performed (Table 6).

In patients with PAPS, none of the investigated variables were demonstrated to be independently associated with FMD (%).

Anti–Apo A-I were detected in 33 of 43 (76.7%) patients with SLE, in 10 of 16 (62.5%) patients with PAPS but in none of the healthy controls (P=0.004 and P=0.001 versus controls, respectively). Moreover, median anti–Apo A-I optical density was significantly higher in patients with SLE (0.17±0.13, P<0.0001) and in patients with PAPS (0.21±0.18, P<0.0001) than in healthy controls (0.03±0.05). No significant correlation was found among anti–Apo A-I and Apo A-I plasma levels or traditional and disease-related cardiovascular risk factors.

Discussion
The main result of this study is the demonstration that a significant percentage of patients with SLE and PAPS show a β2GPI-specific T cell reactivity, which is significantly associated with IMT.

The role of β2GPI in the pathogenesis of atherosclerosis has been demonstrated in humans and in experimental models. In murine models, β2GPI was detected within early atherosclerotic lesions where it was expressed intracellularly and extracellularly.48 β2GPI has also been detected in human atherosclerotic plaques by immunostaining studies showing that β2GPI and aβ2GPI colocalize with oxidized low-density lipoproteins (ox-LDL). 45,49 Furthermore, immunization of Apo-E–deficient mice with β2GPI resulted in an enhancement of atherosclerosis.50 In an experimental model of LDL-receptor–deficient mice, β2GPI-specific cellular immunity showed a central role in promoting atherogenesis.48 When injected with lymphocytes from β2GPI-immunized animals, mice displayed fatty streaks larger than those shown in mice injected with lymphocytes from albumin-immunized controls; T-cell–depleted splenocytes were unable to promote lesion formation, thus suggesting a primary role for T-lymphocytes in mediating atherosclerosis.48

We have recently detected β2GPI-specific T-lymphocytes in the plaque and in the peripheral blood of patients with carotid atherosclerosis.47 In the present study, we have demonstrated

| Table 2. Comparison of Traditional Cardiovascular Risk Factors Among the Study Groups |
|-----------------------------------|---|---|---|---|
| SLE (a) | PAPS (b) | Controls (c) |
| n=50 | n=18 | n=25 |
| **BMI** | | | |
| mean±SD | 24.4±3.8 | 25.5±3.3 | 22.2±3.1 |
| **Systolic blood pressure, mmHg**, mean±SD | 131.1±22.7 | 126.6±15.2 | 114.8±15.9 |
| **Diastolic blood pressure, mmHg**, mean±SD | 83±9.8 | 78.8±10.9 | 73.4±9.1 |
| Diabetes mellitus, n (%) | 2/50 (4) | 1/18 (5.5) | 0/25 |
| Current Smoking, n (%) | 9/50 (18) | 2/18 (11) | 8/25 (32) |
| Family history of cardiovascular disease, n (%) | 9/50 (18) | 3/18 (16.7) | 1/25 (4) |
| Total cholesterol, mg/dL, mean±SD | 182.2±41.4 | 190.7±23.8 | 203.1±46.5 |
| LDL-C, mg/dL, mean±SD | 109.1±30.4 | 121.6±19.2 | 126.5±43 |
| HDL-C, mg/dL, mean±SD | 52.3±16.3 | 48.6±15.2 | 58.4±10.3 |
| Triglycerides, mg/dL, mean±SD | 104.5±49.6 | 102±58.9 | 91.1±47.5 |
| Apolipoprotein A-I, mg/dL, mean±SD | 124.6±24.5 | 149.7±19.5 | 169.4±16.6 |
| Apolipoprotein B, mg/dL, mean±SD | 103.2±25 | 108.3±25.2 | 93.2±23.7 |

BMI indicates body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; NS, not significant; PAPS, primary antiphospholipid syndrome; and SLE, systemic lupus erythematosus.
the presence of a β2GPI-specific T cell reactivity in a significant percentage of patients with SLE and PAPS. In response to β2GPI, proliferating PBMC produced higher amount of IFN-γ than nonproliferating ones. By secreting proinflammatory cytokines, such as IFN-γ as well as by facilitating aPL production, β2GPI-specific T cells seem to play a pathogenic role. The reason why β2GPI-specific autoreactivity and the associated endothelial dysfunction are triggered only in a specific group of patients has still to be clarified. The antigenicity of β2GPI in patients with SLE and PAPS may be explained by the structural modifications of the protein because of chronic inflammation and oxidative stress, with the consequent generation of new antigenic epitopes. Another possible explanation could be the binding of β2GPI to ox-LDL that may facilitate the presentation of β2GPI epitopes by macrophage/dendritic cells to autoreactive T cells. As a matter of fact, β2GPI and aβ2GPI have already been detected within atherosclerotic plaques, and β2GPI immunization of animal models has demonstrated the role of this glycoprotein in plaque progression. It is well known that proatherogenic lipid modifications generate ox-LDL, which represent the main lipoproteins within the atherosclerotic lesion; once oxidized, LDL can attract macrophages and T-lymphocytes into the arterial wall thus perpetuating atherosclerotic plaque progression. Ox-LDLs have highly inflammatory and immunogenic properties. In vitro, ox-LDLs, but not native LDL, bind β2GPI and aβ2GPI autoantibodies; these immune complexes (ox-LDL/β2GPI/antibody) have been found in patients with SLE.

Table 3. Cytokine Secretion in Supernatants From PBMC Samples Responding or Not to β2GPI in Proliferation Assay

<table>
<thead>
<tr>
<th>PBMC Samples</th>
<th>n</th>
<th>Interferon-γ pg/mL, median (range)</th>
<th>Interleukin-4 pg/mL, median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responding patients</td>
<td>15</td>
<td>204.2 * (74.7–2148.4)</td>
<td>13.3 (0–66)</td>
</tr>
<tr>
<td>Nonresponding patients</td>
<td>34</td>
<td>93.05 (48.3–911.2)</td>
<td>10.1 (0–83.5)</td>
</tr>
<tr>
<td>Controls</td>
<td>23</td>
<td>113 (51.9–339.9)</td>
<td>11.7 (0–102.6)</td>
</tr>
</tbody>
</table>

*IFN-γ, responding patients vs nonresponding patients P<0.04. β2GPI indicates β2 glycoprotein I; and PBMCs, peripheral blood mononuclear cells.

Table 4. Comparison of IMT and FMD in Study Populations

<table>
<thead>
<tr>
<th></th>
<th>SLE (a)</th>
<th>PAPS (b)</th>
<th>Controls (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=50</td>
<td>n=18</td>
<td>n=25</td>
<td></td>
</tr>
<tr>
<td>Mean IMT, mm mean±SD</td>
<td>0.69±0.12</td>
<td>0.70±0.17</td>
<td>0.63±0.10</td>
</tr>
<tr>
<td>Maximal IMT, mm mean±SD</td>
<td>0.77±0.12</td>
<td>0.77±0.18</td>
<td>0.60±0.10</td>
</tr>
<tr>
<td>Absolute FMD, cm mean±SD</td>
<td>0.03±0.03</td>
<td>0.05±0.04</td>
<td>0.06±0.03</td>
</tr>
<tr>
<td>FMD, % mean±SD</td>
<td>6.5±6.6</td>
<td>13.4±12.3</td>
<td>14.4±9.2</td>
</tr>
</tbody>
</table>

FMD indicates flow-mediated dilatation; IMT, intima-media thickness; NS, not significant; PAPS, primary antiphospholipid syndrome; and SLE, systemic lupus erythematosus.

Figure 1. Dot plot of proliferative response to β2 glycoprotein I (β2GPI) of peripheral blood mononuclear cell (PBMC) samples from patients with primary antiphospholipid syndrome (PAPS), patients with systemic lupus erythematosus (SLE), and healthy controls. Solid lines represent mean values; dotted line represents the cutoff value.
SLE and APS where they could, at least partially, account for the accelerated atherosclerotic process. It has also been shown that anti-β2GPI and anti–ox-LDL/β2GPI are associated with an adverse outcome in patients with coronary artery disease. Furthermore, in a previous study, anti-β2GPI IgG–ox-LDL complexes have been shown to be predictive for intima-media thickness in patients with APS. Other studies showed that IgG specific for β2GPI–oxLDL complexes has an important role on antigen presentation via Fcγ receptor I thus promoting the activation of β2GPI-specific T cells. A schematic drawing, depicting the signaling transduction pathway triggered by β2GPI/anti-β2GPI on innate immune cells (leading to Th1 response and endothelial cell activation) is reported in Figure II in the online-only Data Supplement.

Increasing evidence suggests that early atherosclerosis in SLE is associated both with traditional cardiovascular risk factors and nontraditional disease–related risk factors. According to previous studies, we demonstrated an increase of carotid IMT and a decrease of FMD in patients with SLE, both correlating with traditional and disease-related risk factors. Compared with controls, mean carotid IMT was also found to be increased in patients with PAPS although it did not reach a statistically significant difference. These results agree with those reported in other studies on patients with PAPS where carotid IMT resulted higher than in age- and sex-matched healthy controls.

As reported by others, patients with SLE showed a higher prevalence of traditional cardiovascular risk factors such as hypertension, body mass index, low HDL-cholesterol, and Apo A-1 plasma levels when compared with controls. Also patients with PAPS presented a significantly higher body mass index and systolic blood pressure and lower Apo A-1 and HDL-cholesterol than controls. The suggested cross-reactivity between aCL and anti-Apo-A-I and anti-HDL could play a role in the decreased Apo A-1 and HDL-cholesterol in patients with PAPS. However, in the present study, we did not find any correlation between anti–Apo A-1 serum levels and traditional disease–related cardiovascular risk factors.

In our SLE population, SDI was the only variable to be independently related to IMT. Such a correlation has already been detected in previous studies. Overall, these findings support the role of chronic inflammation in the atherosclerotic vascular damage.

Mean carotid IMT in patients with PAPS was comparable with that observed in patients with SLE. Multivariate analysis in our patients with PAPS showed a correlation between IMT and anti-β2GPI IgM and age. This result highlighted once again the role of aPL, mainly of anti-β2GPI, in the pathogenesis of atherosclerotic disease. In a previous study on patients with PAPS, aCL IgG titer had been demonstrated as an independent predictor of IMT.

In our cohort, FMD was significantly lower in SLE than in patients with APS and controls. Univariate analysis demonstrated that FMD inversely correlated with total triglycerides and cholesterol plasma levels, but only the latter was independently related to FMD in multivariate analysis. Direct correlation of FMD with cumulative glucocorticoid dosage has also been demonstrated in multivariate analysis, suggesting that a more aggressive therapy could prevent endothelial dysfunction in patients with SLE. The effects of the glucocorticoids on atherosclerotic process are still debated. Indeed, some authors have suggested that glucocorticoids could play a role in the development of atherosclerosis; on the contrary, glucocorticoids are undoubtedly anti-inflammatory molecules that can modulate the disease-related risk factors.

Another result of our study is that β2GPI expression and β2GPI/TF coexpression on monocyte surface were higher in patients with SLE and PAPS than in controls. This result confirms our previous data in another series of patients although no correlations between β2GPI and TF expression on monocytes and FMD or IMT have been identified.

### Table 5. Comparison of IMT and FMD Between Secondary and Primary APS

<table>
<thead>
<tr>
<th></th>
<th>Secondary APS</th>
<th>Primary APS</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean IMT, mm</td>
<td>0.73±0.13</td>
<td>0.70±0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Maximal IMT, mm</td>
<td>0.78±0.13</td>
<td>0.77±0.18</td>
<td>NS</td>
</tr>
<tr>
<td>Absolute FMD, cm</td>
<td>0.02±0.01</td>
<td>0.05±0.04</td>
<td>P=0.047</td>
</tr>
<tr>
<td>β2GPI IgM</td>
<td>6.1±6.4</td>
<td>13.4±12.3</td>
<td>P=0.02</td>
</tr>
</tbody>
</table>

### Table 6. Independent Predictors of Mean IMT and FMD (%) in SLE and PAPS Patients Generated by a Stepwise Multiple Regression Analysis

<table>
<thead>
<tr>
<th>Patients</th>
<th>Independent Variable</th>
<th>Predictor</th>
<th>β</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>mIMT</td>
<td>SDI</td>
<td>0.469</td>
<td>0.001</td>
</tr>
<tr>
<td>n=50</td>
<td>FMD (%)</td>
<td>Total cholesterol</td>
<td>-0.627</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cumulative prednisone dose</td>
<td>0.357</td>
<td>0.014</td>
</tr>
<tr>
<td>PAPS</td>
<td>mIMT</td>
<td>anti-β2GPI IgM</td>
<td>0.681</td>
<td>0.0001</td>
</tr>
<tr>
<td>n=18</td>
<td></td>
<td>Age</td>
<td>0.380</td>
<td>0.004</td>
</tr>
</tbody>
</table>

aβ2GPI indicates anti-β2 glycoprotein I antibodies; FMD, flow-mediated dilatation; mIMT, mean intima-media thickness; PAPS, primary antiphospholipid syndrome; SLE, systemic lupus erythematosus; and SDI, systemic lupus international collaborative clinics/American College of Rheumatology damage index.
extrapolation and applicability of the results to all patients with these autoimmune diseases should be carefully considered.

Early noninvasive subclinical atherosclerosis evaluation calls for further studies and suggests the need for more assessment focusing on the risk factors, preventive interventions, and therapeutic strategies.

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Disclosures

None.

References


Cardiovascular events represent one of the major causes of morbidity and mortality in patients with SLE and APS. Classical risk factors do not completely explain the excess of cardiovascular morbidity, and therefore disease-related markers of atherosclerosis are needed to identify patients at higher risk and to elaborate preventive strategies. This study demonstrates the presence of a β2GPI-specific T cell reactivity in a significant percentage of patients with SLE and APS and its correlation with IMT. β2GPI-specific T cell response might represent an additional factor when evaluating the risk of atherosclerosis in patients with SLE and APS.
Subclinical Atherosclerosis in Systemic Lupus Erythematosus and Antiphospholipid Syndrome: Focus on β2GPI-Specific T Cell Response

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Supplemental Figure I

Interferon-γ (A) and Interleukin-4 (B) secretion in supernatants from peripheral blood mononuclear cell samples responding or not to β2GPI in the proliferation assay. Solid lines represent arithmetic mean values.
Supplemental Figure II

Signalling pathway induced by β2GPI/aβ2GPI. Schematic drawing depicting the signalling transduction pathway triggered by β2GPI/aβ2GPI on innate immune cells, leading to Th1 response and endothelial cell activation. β2GPI/aβ2GPI triggering, through activation of TLRs, induces MyD88-IRAK phosphorylation, which in turn activates TRAF6-TAK1 pathway. This latter pathway induces both NF-kB translocation into the nucleus with consequent release of proinflammatory factors and ERK activation with consequent cell proliferation. Released cytokines, chemokines, adhesion molecules and tissue factor contribute to endothelial cell activation.

aβ2GPI: anti-β2GPI antibodies; TLR: toll-like receptor; IRAK: IL-1 receptor associated kinase; NF-kB: nuclear factor-kappa B; ERK: extracellular signal-regulated kinases; JNK: c-Jun N-terminal kinases; AP-1: activator protein 1.
MATERIALS AND METHODS

Study population
We studied 50 consecutive patients with SLE and 18 consecutive patients with PAPS attending the Lupus Clinic of the Rheumatology Division of “Sapienza” University of Rome, Italy.

Nineteen SLE patients presented secondary APS. SLE was diagnosed according to the American College of Rheumatology revised criteria, and PAPS according to the Sydney revised criteria. Twenty five healthy controls comparable for sex and age served as control group. At each visit, patients underwent a complete physical examination; we registered demographic data, past medical history with date of diagnosis, traditional risk factors for atherosclerosis, co-morbidities, and past and present treatments and provided a written informed consent. Clinical and laboratory data were collected in a standardized, computerized, and electronically-filled form. A fasting blood sample was taken from each patient at the time of clinical evaluation. The study was approved by the local ethical committee of “Policlinico Umberto I”, Rome.

Traditional and disease related risk factors for atherosclerosis
The following variables were considered: age, sex, family history of cardiovascular disease (defined as occurrence of myocardial infarction, stroke, peripheral vascular disease or sudden death in first-degree relatives before the age of 65 years for men and 55 years for women), hypertension (systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg on two or more visits or the use of antihypertensive medications), cigarette smoking (current smokers, former-smokers), diabetes mellitus (defined according to WHO criteria), body mass index (BMI) (kg/m²); total cholesterol (hypercholesterolaemia, if >200 mg/dl) and total triglycerides (hypertriglyceridaemia, if >200 mg/dl).

SLE disease activity was assessed with the SLEDAI (SLE Disease Activity Index) while chronic damage was measured with the Systemic Lupus International Collaborating Clinics/ACR Damage Index score (SLICC). Cumulative clinical manifestations of SLE and PAPS were recorded. Cumulative prednisone dose was calculated.

Laboratory measurements
Blood samples were collected early in the morning, after an overnight fast, in EDTA-containing tubes. Plasma was obtained by centrifugation at 4°C and added with EDTA (0.04%), NaNO₃ (0.05%) and PMSF (0.015%) to prevent lipoprotein modification. Some aliquots were immediately used for glucose and lipid determinations and others were stored at –80°C for the other measurements. Plasma concentration of total cholesterol (TC), TG and HDL-C were determined as previously described. LDL-C was calculated by using the Friedewald’s equation. Plasma levels of Apolipoprotein B and Apolipoprotein A-I (Apo A-I) were determined by an immunoturbidimetric method, as reported.

Anti-cardiolipin (aCL) and anti-β2GPI IgG and IgM antibodies were detected by ELISA (Diamedix, Miami, FL, USA) in accordance with the manufacturer’s instructions. Anti-Apolipoprotein A-I antibodies (anti-Apo A-I) were detected by ELISA (MyBioSource, Inc. San Diego, USA) in accordance with the manufacturer’s instructions. All assays were performed in duplicate. A positive control and several normal human sera were run in the same assay to confirm the specificity of the results.

Carotid ultrasound examination
Carotid IMT was evaluated using duplex Doppler transductor of 7.5–10MHz for the real time imaging. Patients were examined in the supine position, with the head turned 45° away from the side being scanned. The scanning was performed at right and left carotid
A total of three IMT measurements on each side were taken at the following points: common carotid artery (10 mm below the bulb), carotid bifurcation and internal carotid artery (1–2 cm distal to the junction). For each study the following parameters were assessed: I) intima-media wall thickness (IMT) in all territories (IMT of 0.9 mm or less was considered normal); II) mean IMT (m-IMT), as the mean of the three IMT measurements on each side; III) maximum IMT (M-IMT), as the highest IMT value found among the six segments studied; IV) the presence of plaque, defined as a focal or diffuse thickness of 1.2 mm or more. Plaque analysis included location, number and maximum height.

**Assessment of flow mediated dilatation (FMD)**

FMD in response to reactive hyperemia (endothelium dependent vasodilatation) was evaluated on brachial artery by employing a high-resolution B-mode Doppler (ATL HDI 5000 with a 7.4-MHz linear-array transducer) and following the guidelines published by the International Brachial Arterial Reactivity Task Force. In brief, all subjects were studied between 8 and 11 AM after a 12-hour overnight fast in a quiet and stable temperature environment. A straight, non branching segment of the brachial artery 5-15 cm above the antecubital fossa was identified by a B-mode longitudinal scan. Vessel diameter was recorded in a segment with clear anterior and posterior intimal interfaces between the lumen and vessel wall by a blinded reader at rest and during reactive hyperemia. Brachial artery diameter was measured offline by an automatic edge-detection system. A blood pressure cuff was then inflated around the forearm to a supra-systolic pressure (at least 50 mm Hg above the systolic pressure to occlude arterial inflow) for the standardized length of time of 5 minutes. Measurement of the maximum diameter of the artery was taken 45 to 60 seconds after the cuff release. Absolute FMD was expressed as: [postdeflation diameter – resting diameter]; FMD relative values were also calculated as a percent change from the baseline diameter as follows: 100% x [(postdeflation diameter - resting diameter)/resting diameter].

**β2GPI and Tissue Factor (TF) expression on human monocytes.**

Human peripheral blood mononuclear cells (PBMC) were isolated from freshly heparinized blood by Lymphoprep (Nycomed AS Pharma Diagnostic Division, Oslo, Norway) density-gradient centrifugation and washed three times in phosphate buffered saline (PBS), pH 7.4. Monocytes were isolated by counterflow centrifugal elutriation according to the Current Protocols in Immunology. Monocyte populations were washed with RPMI 1640 and placed in serum-free medium, containing 5 mM insulin, 5 mM transferrine, 100 U/ml penicillin, 100 mg/ml streptomycin, 250 pg/ml fungizone and 2 mM L-glutamine (obtained from Gibco BRL, Life Technologies Italia srl, Milan, Italy) for 24 h at 37°C in a humidified 5% CO2 atmosphere. The purity of monocyte preparation was checked by cytofluorimetric analysis by FITC-conjugated anti-CD14 (IgG2a k isotype, DAKO S.p.A.). All preparations contained up to 80% of anti-CD14 positive cells.

Indirect immunofluorescence was performed to analyse β2GPI expression on cell plasma membrane of monocytes. One x 10⁶ cells were fixed in 4% formaldehyde/PBS for 1 h at 4°C. After washing three times with PBS, cells were incubated with a rabbit polyclonal anti-human β2GPI (Affinity Biologicals Inc., Ancaster, Canada) (28), in PBS/1% BSA, for 1h at 4 °C. Phycoerythrin (PE)-conjugated anti-goat IgG (y-chain specific, Sigma) were then added and incubated at 4 °C for 30 min After washing with PBS, cells were incubated with FITC-conjugated monoclonal antibodies (MoAb) anti-CD14 (Sigma) or anti-human tissue factor (American Diagnostica Inc., Greenwich, CT, USA) for 45 min at 4 °C. For isotype control, FITC-conjugated mouse IgG (Sigma) were employed. The fluorescence intensity was analysed with a Becton Dickinson cytometer. Cells were gated on the basis of forward angle light scatter and 90° light scatter parameters.
β2GPI specific proliferation assay

Cell proliferation was assessed in PBMC samples from 37 patients with SLE, 12 patients with PAPS and 23 healthy controls, isolated as previously described. Triplicate cultures of PBMC (1x10^6 cells/ml) were stimulated for 5 days with human β2GPI (Calbiochem, USA 25 μg/ml) or left unstimulated. Samples stimulated with phytohaemagglutinin (Burroughs Wellcome Co., Beckenham, UK, - 2 μg/ml) or human serum albumin (HSA, Sigma-Aldrich; 10 μg/ml) were set up as positive and negative controls of proliferation assay. Preliminary experiments demonstrated that the used β2GPI concentration determined effective cell stimulation. Endotoxin contamination in antigen preparations, as determined by the quantitative chromogenic Limulus amebocyte lysate assay (QCL-1000, BioWhittaker, Walkersville, MD) was <0.03 endotoxin units/μg of protein. Cell proliferation was assessed by adding 0.5 μCi ³H-methyl-thymidine (specific activity 1 mCi/mmol) (Amersham Life Science, Buckinghamshire, UK) to each well. Cells were harvested after 18 hours on glass fiber filter paper (Wallac, EG & G Company, Turku, Finland), using an automatic cell harvester (Harvester 96, MACH III M, TOMTEC, Orange, CT, USA), and analyzed in a β counter (1450 Microbeta Plus, Wallac). Net counts per minute (cpm) of triplicate cultures were determined and the proliferative response was expressed as stimulation indices (SI, defined as the ratio between the mean cpm in stimulated and unstimulated cultures). Mean SI in healthy subjects plus 2 standard deviations was taken as the threshold level for a positive immunoreaction.

IFN-γ and IL-4 concentrations in supernatants from PBMC samples stimulated with β2GPI were quantified with commercially available ELISA sets (OptEIA set, BD Biosciences, CA, USA) as recommended by the manufacturer. The assay ranges were 4.7-300 pg/ml for IFN-γ; 7.8-500 pg/ml for IL-4.

Statistical analysis

We used version 13.0 of the SPSS statistical software package. Values are reported as mean ± standard deviation (S.D.) unless otherwise stated. Differences between numeric variables were tested using the Mann-Whitney U test. Correlation was tested by Spearman’s rank-order or Pearson’s correlation coefficient. For comparison of categorical variables or percentages, we used χ² and Fisher’s exact tests when appropriate. Multiple linear regression analysis was used to test for independent associations between FMD, IMT and various factors. P values less than 0.05 were considered significant.

References


