

# Interleukin-17/Interleukin-21 and Interferon- $\gamma$ producing T cells specific for $\beta$ 2 Glycoprotein I in atherosclerosis inflammation of systemic lupus erythematosus patients with antiphospholipid syndrome

Marisa Benagiano,<sup>1</sup> Maria Orietta Borghi,<sup>2,3</sup> Jacopo Romagnoli,<sup>4</sup> Michael Mahler,<sup>5</sup> Chiara Della Bella,<sup>1</sup> Alessia Grassi,<sup>1</sup> Nagaja Capitani,<sup>1</sup> Giacomo Emmi,<sup>1,6</sup> Arianna Troilo,<sup>1</sup> Elena Silvestri,<sup>1</sup> Lorenzo Emmi,<sup>6</sup> Heba Alwaisri,<sup>1</sup> Jacopo Bitetti,<sup>1</sup> Simona Tapinassi,<sup>1</sup> Domenico Prisco,<sup>1,6</sup> Cosima Tatiana Baldari,<sup>7</sup> Pier Luigi Meroni<sup>2\*</sup> and Mario Milco D'Elcios<sup>1,6\*</sup>

<sup>1</sup>Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy; <sup>2</sup>IRCCS, Istituto Auxologico Italiano, Laboratory of Immunorheumatology, Cusano Milanino, Italy; <sup>3</sup>Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy; <sup>4</sup>Department of Surgery, Rome Catholic University, Rome, Italy; <sup>5</sup>Inova Diagnostics La Jolla, La Jolla, CA, USA.; <sup>6</sup>Internal Interdisciplinary Medicine, Lupus Clinic, AOU Careggi, Florence, Italy and <sup>7</sup>Department of Life Sciences, University of Siena, Siena, Italy

## ABSTRACT

Systemic lupus erythematosus is frequently associated with antiphospholipid syndrome. Patients with lupus-antiphospholipid syndrome are characterized by recurrent arterial/venous thrombosis, miscarriages, and persistent presence of autoantibodies against phospholipid-binding proteins, such as  $\beta$ 2-Glycoprotein I. We investigated the cytokine production induced by  $\beta$ 2-Glycoprotein I in activated T cells that infiltrate *in vivo* atherosclerotic lesions of lupus-antiphospholipid syndrome patients. We examined the helper function of  $\beta$ 2-Glycoprotein I-specific T cells for tissue factor production, as well as their cytolytic potential and their helper function for antibody production. Lupus-antiphospholipid syndrome patients harbor *in vivo* activated CD4<sup>+</sup> T cells that recognize  $\beta$ 2-Glycoprotein I in atherosclerotic lesions.  $\beta$ 2-Glycoprotein I induces T-cell proliferation and expression of both Interleukin-17/Interleukin-21 and Interferon- $\gamma$  in plaque-derived T-cell clones.  $\beta$ 2-Glycoprotein I-specific T cells display strong help for monocyte tissue factor production, and promote antibody production in autologous B cells. Moreover, plaque-derived  $\beta$ 2-Glycoprotein I-specific CD4<sup>+</sup> T lymphocytes express both perforin-mediated and Fas/FasLigand-mediated-cytotoxicity. Altogether, our results indicate that  $\beta$ 2-Glycoprotein I is able to elicit a local Interleukin-17/Interleukin-21 and Interferon- $\gamma$  inflammation in lupus-antiphospholipid syndrome patients that might lead, if unabated, to plaque instability and subsequent arterial thrombosis, suggesting that the T helper 17/T helper 1 pathway may represent a novel target for the prevention and treatment of the disease.

## Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that is frequently associated with antiphospholipid syndrome (APS) characterized by recurrent vascular thrombosis and pregnancy morbidities associated with the persistent presence of autoantibodies against phospholipid-binding proteins, namely antiphospholipid antibodies (aPL), such as  $\beta$ 2-glycoprotein I ( $\beta$ 2GPI).<sup>1</sup> Besides its role in the acquired pro-coagulant diathesis, aPL have been also associated with accelerated atherosclerosis to explain cardiovascular manifestations of the syndrome.<sup>2-4</sup> An accelerated atherosclerosis in SLE was first demonstrated in 1975 by



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## Correspondence:

MARIO MILCO D'ELIOS  
mariomilco.delios@unifi.it/delios@unifi.it

PIERLUIGI MERONI  
pierluigi.meroni@unimi.it

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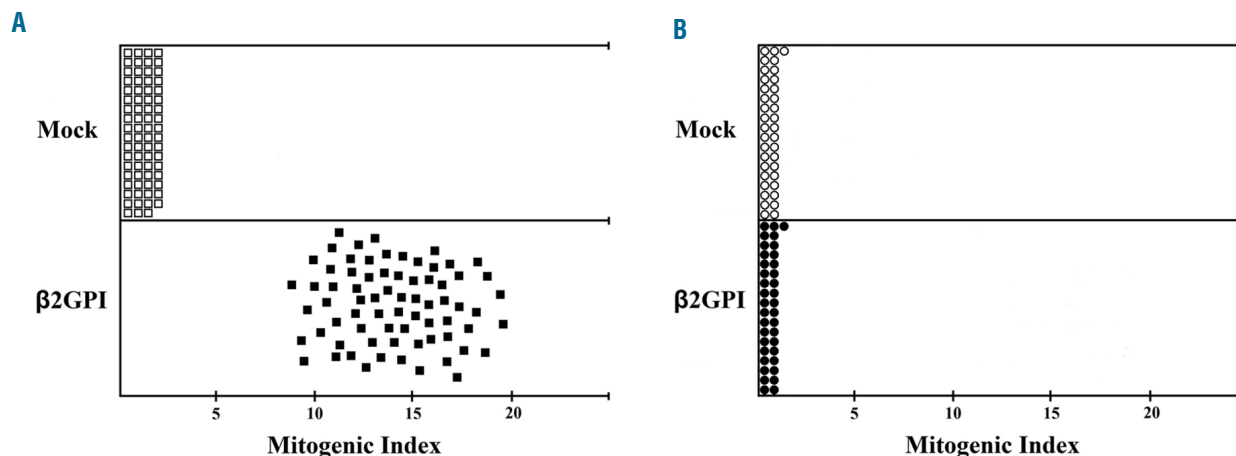
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**Figure 1.** Antigen specificity of atherosclerotic plaque CD4<sup>+</sup> T and CD8<sup>+</sup> T-cell clones obtained from systemic lupus erythematosus patients with antiphospholipid syndrome. Both CD4<sup>+</sup> T- and CD8<sup>+</sup> T-cell clones were tested for antigen-specificity. T-cell clones were analyzed for their responsiveness to  $\beta$ 2GPI (10 nM) (■), or medium (□) by measuring [<sup>3</sup>H]thymidine uptake after 60 hours of co-culture with irradiated autologous peripheral blood mononuclear cells. Seventy-one out of 297 CD4<sup>+</sup> T-cell clones proliferated in response to  $\beta$ 2GPI and are shown in (A). None of the 37 CD8<sup>+</sup> T-cell clone proliferated to  $\beta$ 2GPI (B).

Bulkley *et al.*<sup>5</sup> in a necroscopic study, that was further confirmed by Urowitz *et al.*<sup>6</sup>

Many studies showed that SLE is associated with coronary heart disease and atherosclerosis;<sup>7-9</sup> an important prospective study demonstrated that SLE patients have an accelerated progression of carotid plaque formations compared to non-lupus controls.<sup>10</sup> SLE patients have a reduced life expectancy mainly due to the increased prevalence of cardiovascular diseases. Incidence of major cardiovascular events is 2.5 times higher in SLE patients compared to the general population. Compared to healthy subjects, SLE women, aged 35-44 years, have a 50 times increased risk of myocardial infarction and accelerated atherosclerosis, that is a well recognized comorbidity in SLE.<sup>11,12</sup>

Atherosclerosis is a multifactorial disease for which a number of different pathogenic mechanisms have been proposed. In addition to classical risk factors, in the last two decades, attention has been focused on inflammatory processes.<sup>13,14</sup> Observations in humans and animals suggest that atherosclerotic plaques derive from specific cellular and molecular mechanisms that can be ascribed to an inflammatory disease of the arterial wall, the lesions of which consist of activated macrophages and T lymphocytes. If inflammation continues unabated, it results in an increased number of plaque-infiltrating macrophages and T cells, which contribute to the remodeling of the arterial wall, eventually favoring plaque instability and rupture.<sup>15</sup> Within the T-cell population infiltrating the plaque, most cells are activated CD4<sup>+</sup> T helper (Th) 1 and Th17 cells expressing HLA-DR and the interleukin (IL)-2 receptor (CD25).<sup>16,17</sup>

Current evidence indicates that autoimmunity can be detected within the atherosclerotic lesions.<sup>18</sup> Accordingly, self-phospholipids, such as oxidized low-density lipoprotein (oxLDL) and human heat shock proteins, drive T-cell inflammation in atherosclerotic patients.<sup>19,20</sup> However, the multifactorial nature of atherosclerosis suggests that a larger number of autoantigens might be involved.

It has been hypothesized that the development of an anti- $\beta$ 2GPI-specific response in the target organ may con-

tribute to atherothrombosis in SLE-APS patients. This hypothesis is largely based on the  $\beta$ 2GPI presence in human atherosclerotic plaques<sup>21,22</sup> and on the enhanced fatty streak formation in transgenic atherosclerosis-prone mice immunized with  $\beta$ 2GPI.<sup>23,24</sup> Moreover,  $\beta$ 2GPI-reactive T cells have also been found to promote early atherosclerosis in LDL receptor deficient mice.<sup>25</sup>

In this study, we demonstrate that, in SLE-APS patients, both IL-17 and IFN- $\gamma$  are secreted by atherosclerotic plaques infiltrating Th cells in response to  $\beta$ 2GPI, and suggest that  $\beta$ 2GPI drives a local Th17/Th1 inflammatory response, which can be responsible for plaque instability and rupture, leading to atherothrombosis.

## Methods

A detailed description of the methods is available in the *Online Supplementary Appendix*.

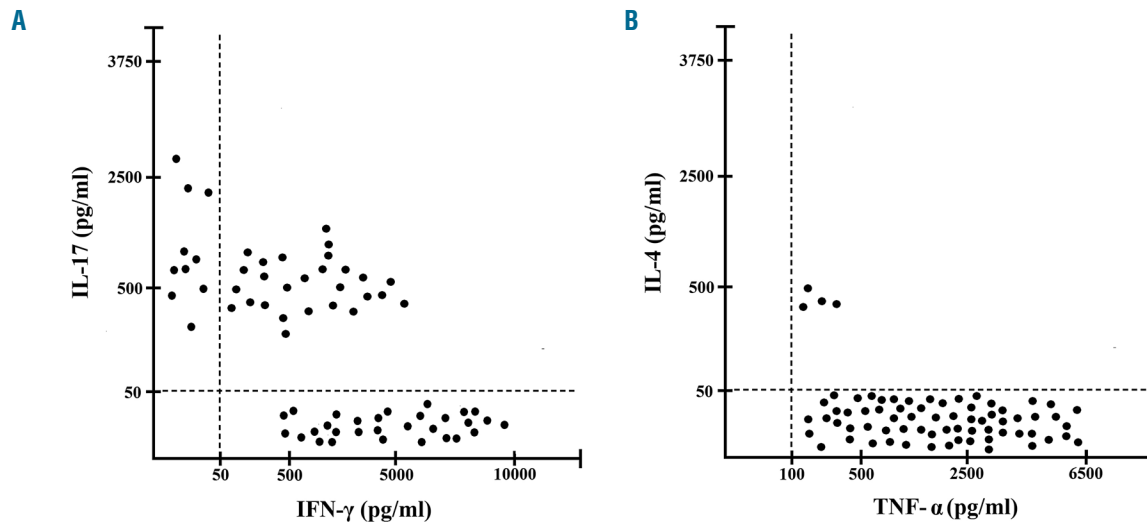
## Reagents

Human  $\beta$ 2GPI was purified as described.<sup>26</sup> We ruled out the presence of contaminants by a limulus test. The human  $\beta$ 2GPI used was with a limulus test and resulted negative throughout the whole study.

## Patients

Upon approval of the local Ethical Committee, the following patients were enrolled in the study: ten patients (10 females; mean age 51 years, range 42-56 years) with SLE-APS, ten aPL negative patients (10 females; mean age 51 years, range 43-55 years), five SLE aPL-positive patients (5 females; mean age 49 years, range 44-53 years), and five SLE aPL-negative patients (5 females; mean age 50 years, range 44-56 years); all were affected by carotid atherosclerotic arteriopathy. The carotid plaques were obtained by endarterectomy from each patient. The clinical information of each patient is reported in *Online Supplementary Tables S1-S4*.

All patients studied (SLE-APS, SLE aPL-positive, SLE aPL-negative, and aPL negative patients) were eligible for vascular surgery. All the SLE aPL-positive patients were affected by SLE but not by



**Figure 2. Cytokine profile of atherosclerotic plaque  $\beta$ 2GPI-specific CD4<sup>+</sup> T-cell clones obtained from systemic lupus erythematosus patients with antiphospholipid syndrome.** The clones were tested for cytokine production (A and B).  $\beta$ 2GPI-specific Th clones were stimulated with  $\beta$ 2GPI and TNF- $\alpha$  and IL-4, IFN- $\gamma$  and IL-17 production was measured in culture supernatants. In unstimulated cultures, levels of TNF- $\alpha$ , IL-4, IFN- $\gamma$  and IL-17 were consistently < 20 pg/mL. CD4<sup>+</sup> T-cell clones producing IFN- $\gamma$ , but not IL-17 nor IL-4 were coded as Th1. CD4<sup>+</sup> T-cell clones producing IL-17, but not IFN- $\gamma$  nor IL-4 were coded as Th17. CD4<sup>+</sup> T-cell clones producing IFN- $\gamma$ , and IL-17, but not IL-4 were coded as Th17/Th1. CD4<sup>+</sup> T-cell clones producing TNF- $\alpha$  and IL-4, but not IL-17 were coded as Th0.

APS, although they were positive for aPL, with serum anti- $\beta$ 2GPI, anti-cardiolipin antibodies or with positivity for LA. All SLE aPL-neg patients were affected by SLE but not by APS, and they were triple negative for serum aPL, such as anti- $\beta$ 2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant.

#### Anti-phospholipid antibody detection

The detection of aCL and a $\beta$ 2GPI in patient sera, and analysis of LA was performed as described elsewhere.<sup>28,29</sup>

#### Generation and characterization of T-cell clones from atherosclerotic plaques inflammatory infiltrates

Carotid specimens, obtained by endoarterectomy, were investigated in both SLE-APS and in aPL negative patients under the same experimental conditions. Specimens were then disrupted, and single T cells were cloned under limiting dilution, as described.<sup>16</sup> To assess their phenotype profile, T-cell clones were screened by flow cytometry with fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8 on a BD FACSCanto II (BD Bioscience), using the FACS Diva 6.1.3. software. The repertoire of the TCR V $\beta$  chain of  $\beta$ 2GPI-specific Th clones was analyzed with a panel of mAb specific to the following: V $\beta$ 1, V $\beta$ 2, V $\beta$ 4, V $\beta$ 5.1, V $\beta$ 5.2, V $\beta$ 5.3, V $\beta$ 7, V $\beta$ 8, V $\beta$ 9, V $\beta$ 11, V $\beta$ 12, V $\beta$ 13.1, V $\beta$ 13.2 and V $\beta$ 13.6, V $\beta$ 14, V $\beta$ 16, V $\beta$ 17, V $\beta$ 18, V $\beta$ 20, V $\beta$ 21.3, V $\beta$ 22, and V $\beta$ 23 (Beckman Coulter); V $\beta$ 6.7 (Gentaur) and V $\beta$ 3.1 (In Vitro Gen). Isotype-matched non-specific Ig were used as negative control. V $\beta$ 10, V $\beta$ 15, and V $\beta$ 19 T-cell receptor typing were investigated by Clontech kit, according to the manufacturer's instructions. Each  $\beta$ 2GPI-reactive CD4<sup>+</sup> T-cell clone was stained by only one of the TCR-V $\beta$  chain-specific monoclonal antibodies, showing a single peak of fluorescence intensity (*Online Supplementary Figure S1*). The cytokine production, the cytotoxicity, the helper functions for antibody and tissue factor production of  $\beta$ 2GPI-specific T-cell clones were performed as described.<sup>16,30,31</sup>

#### Statistical analysis

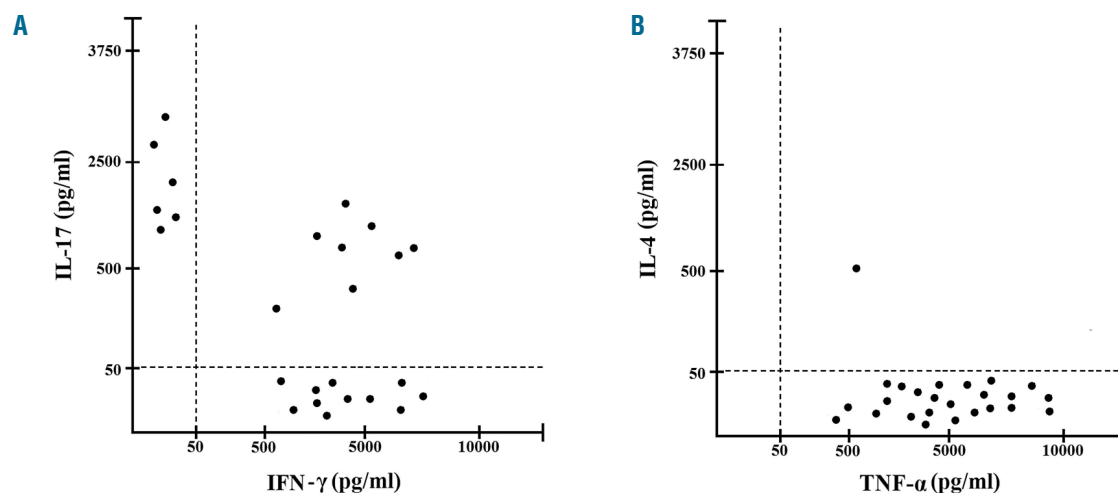
Statistical analyses were performed using Student's *t*-test. *P*<0.05 was considered significant.

## Results

### Atherosclerotic lesions of systemic lupus erythematosus patients with antiphospholipid syndrome and systemic lupus erythematosus patients positive for antiphospholipid antibodies harbor autoreactive $\beta$ 2GPI-specific CD4<sup>+</sup> T-cell clones

Atherosclerotic plaque-infiltrating *in vivo* activated T cells were expanded *in vitro* in an hrIL-2 conditioned medium, subsequently cloned and studied for their phenotypic and functional profile. A total number of 297 CD4<sup>+</sup> and 37 CD8<sup>+</sup> T-cell clones were obtained from atherosclerotic lesions of ten SLE-APS patients. For each patient, CD4<sup>+</sup> and CD8<sup>+</sup> atherosclerotic lesion-derived T-cell clones were assayed for proliferation in response to medium, or  $\beta$ 2GPI. None of the CD8<sup>+</sup> T-cell clones showed proliferation to  $\beta$ 2GPI although they proliferated in response to mitogen stimulation (Figure 1). We have also investigated the amount of  $\beta$ 2GPI-specific T cells present in the peripheral blood of SLE-APS patients and compared it with the one found in atheromas. The proportion of  $\beta$ 2GPI-specific CD4<sup>+</sup> T-cell clones generated from atherosclerotic plaques of SLE-APS patients was 24%, which is remarkably higher than the frequency of  $\beta$ 2GPI-specific T cells found in the peripheral blood of the same patients (between 1:1900 and 1:3400).

Seventy-one (24%) of the 297 CD4<sup>+</sup> T-cell clones generated from SLE-APS atherosclerotic plaque-infiltrating T cells proliferated significantly to  $\beta$ 2GPI (Figure 1). Each SLE-APS patient displayed a comparable percentage of CD4<sup>+</sup> T-cell clones responsive to  $\beta$ 2GPI (*Online Supplementary Table S1*). On the other hand, a total number of 288 CD4<sup>+</sup> and 42 CD8<sup>+</sup> T-cell clones were obtained from atherosclerotic lesions of ten atherothrombotic patients, that were negative for aPL. For each patient, CD4<sup>+</sup> and CD8<sup>+</sup> atherosclerotic lesion-derived T-cell clones were assayed for proliferation in response to medium or  $\beta$ 2GPI. None of the CD4<sup>+</sup> or CD8<sup>+</sup> T-cell clones



**Figure 3. Cytokine profile of atherosclerotic plaque  $\beta$ 2GPI-specific CD4<sup>+</sup> T-cell clones obtained from systemic lupus erythematosus patients positive for antiphospholipid antibodies.** Th clones were tested for cytokine production (A and B).  $\beta$ 2GPI-specific Th clones were stimulated with  $\beta$ 2GPI and TNF- $\alpha$  and IL-4, IFN- $\gamma$  and IL-17 production was measured in culture supernatants. In unstimulated cultures, levels of TNF- $\alpha$ , IL-4, IFN- $\gamma$  and IL-17 were consistently < 20 pg/mL. CD4<sup>+</sup> T-cell clones producing IFN- $\gamma$ , but not IL-17 nor IL-4 were coded as Th1. CD4<sup>+</sup> T-cell clones producing IL-17, but not IFN- $\gamma$  nor IL-4 were coded as Th17. CD4<sup>+</sup> T-cell clones producing IFN- $\gamma$ , and IL-17, but not IL-4 were coded as Th17/Th1. CD4<sup>+</sup> T-cell clones producing TNF- $\alpha$  and IL-4, but not IL-17 were coded as Th0.

derived from the atherosclerotic lesions showed proliferation to  $\beta$ 2GPI (*Online Supplementary Table S2*). A total number of 135 CD4<sup>+</sup> and 21 CD8<sup>+</sup> T-cell clones were obtained from atherosclerotic lesions of five SLE aPL-positive. For each patient, CD4<sup>+</sup> and CD8<sup>+</sup> atherosclerotic lesion-derived T-cell clones were assayed for proliferation in response to medium or  $\beta$ 2GPI. 25 CD4<sup>+</sup> and no CD8<sup>+</sup> T-cell clones derived from the atherosclerotic lesions of SLE aPL-positive patients showed proliferation to  $\beta$ 2GPI (*Online Supplementary Table S3*). A total number of 136 CD4<sup>+</sup> and 30 CD8<sup>+</sup> T-cell clones were obtained from atherosclerotic lesions of five SLE aPL-negative. For each patient, CD4<sup>+</sup> and CD8<sup>+</sup> atherosclerotic lesion-derived T-cell clones were assayed for proliferation in response to medium or  $\beta$ 2GPI. None of the CD4<sup>+</sup> or CD8<sup>+</sup> T-cell clones derived from the atherosclerotic lesions showed proliferation to  $\beta$ 2GPI (*Online Supplementary Table S4*).

All  $\beta$ 2GPI-specific T-cell clones, both those obtained from the atherosclerotic lesions of SLE-APS patients and those obtained from SLE aPL-positive patients, were stimulated with  $\beta$ 2GPI and autologous APC. Then, TNF- $\alpha$  and IL-4, IFN- $\gamma$  and IL-17 production was measured in culture supernatants. Upon antigen stimulation with  $\beta$ 2GPI of the 71  $\beta$ 2GPI-specific T-cell clones obtained from SLE-APS patients, 30 were polarized Th1 clones, 10 Th clones were Th17, 27 Th clones were Th17/Th1, and only 4 were able to produce IL-4 together with TNF- $\alpha$  (Th0 clones) (Figure 2). Upon antigen stimulation with  $\beta$ 2GPI of the 25  $\beta$ 2GPI-specific T-cell clones obtained from SLE aPL-positive patients, 10 were polarized Th1 clones, 6 Th clones were polarized Th17, 8 Th clones were Th17/Th1, and only one was Th0 (Figure 3). T-cell blasts from each of the 71  $\beta$ 2GPI-reactive T-cell clones obtained from atherosclerotic lesions of patients with SLE-APS were further screened by IFN- $\gamma$  and IL-17 ELISPOT in response to  $\beta$ 2GPI. Upon appropriate stimulation, 61 atherosclerotic-derived CD4<sup>+</sup> T-cell clones produced IFN- $\gamma$ , and thirty-seven produced IL-17 (Figure 4). Interestingly, all IL-17-producing  $\beta$ 2GPI-reactive T-cell clones, produce IL-21 (mean $\pm$ SE, 3.3 $\pm$ 0.5 ng/mL per 10<sup>6</sup> T cells) in response to antigen stimulation.

### **$\beta$ 2GPI-specific atherosclerotic lesion-infiltrating T cells help monocyte tissue factor production and procoagulant activity**

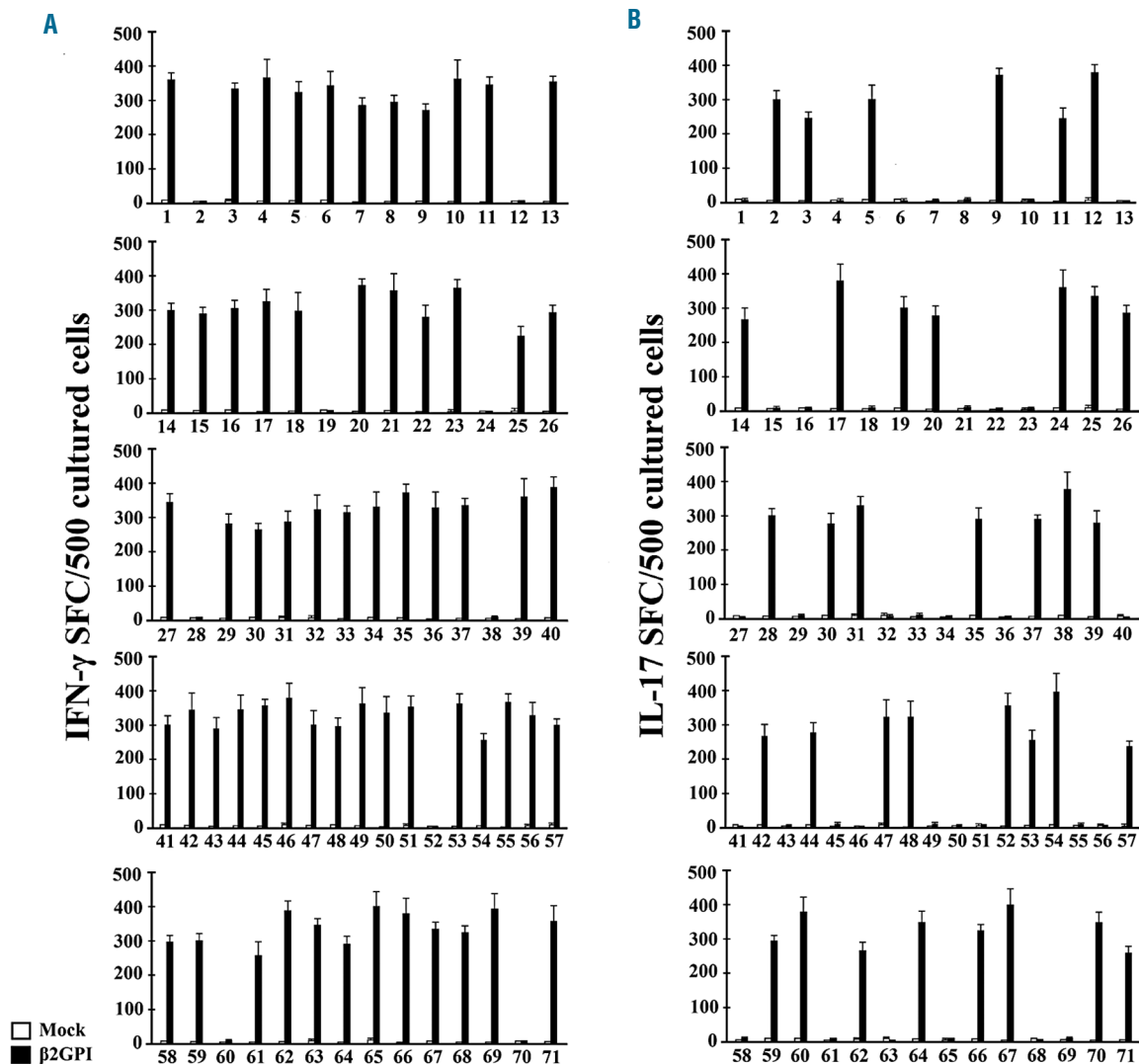
Plaque rupture and consequent thrombosis are crucial complications of atherosclerosis. TF plays a key role in triggering atherothrombotic events being the primary activator of the coagulation cascade. We investigated whether atherosclerotic lesion-infiltrating  $\beta$ 2GPI-specific T cells had the potential to express helper functions for TF production and PCA by autologous monocytes. Antigen-stimulated  $\beta$ 2GPI-specific atherosclerotic lesion-derived T-cell clones were co-cultured with autologous monocytes and levels of TF and PCA were measured. Antigen stimulation resulted in the expression of substantial help for TF (Figure 5A) production and PCA (Figure 5B) by autologous monocytes.

### **Atherosclerotic lesion-derived $\beta$ 2GPI-specific T-cell clones express antigen-dependent help to autologous B cells for Ig production**

T/B-cell interaction is a multistep process resulting in B-cell help depending on the functional commitment of the Th cells involved. So far the ability of SLE-APS-derived  $\beta$ 2GPI-specific T-cell clones to provide B-cell help for Ig synthesis has been investigated. In the absence of the specific antigen, no increase in IgM, IgG, or IgA production above spontaneous levels measured in cultures containing B cells alone was observed. In the presence of  $\beta$ 2GPI and at a T-to-B cell ratio of 0.2 to 1, all of the  $\beta$ 2GPI-specific T-cell clones provided substantial help for Ig production. At a 1:1 T/B cell ratio,  $\beta$ 2GPI-dependent T-cell help for IgM, IgG, and IgA production by B cells was much higher (Figure 6). However, at a 5:1 T/B cell ratio, co-culturing B cells with autologous  $\beta$ 2GPI-specific T-cell clones in the presence of  $\beta$ 2GPI resulted in a much lower Ig synthesis.

### **Atherosclerotic lesion-derived $\beta$ 2GPI-specific T-cell clones display cytotoxic and pro-apoptotic activity**

The cytolytic potential of SLE-APS-derived atherosclerotic lesion-derived  $\beta$ 2GPI-specific autoreactive T-cell



**Figure 4.**  $\beta$ 2GPI driven IFN- $\gamma$  and IL-17 secretion by  $\beta$ 2GPI-specific atherosclerotic plaque derived Th clones from systemic lupus erythematosus patients with antiphospholipid syndrome. Numbers of IFN- $\gamma$  spot-forming cells (SFC) after stimulation of atherosclerotic plaque derived T-cell clones with medium alone, or  $\beta$ 2GPI (A). T-cell blasts from each clone were stimulated for 48 hours (h) with medium alone ( $\square$ ), or  $\beta$ 2GPI ( $\blacksquare$ ), in the presence of irradiated autologous antigen-presenting cell (APC) in ELISPOT microplates coated with anti-IFN- $\gamma$  antibody. IFN- $\gamma$  SFC were then counted by using an automated reader. After specific stimulation, 61 of 71  $\beta$ 2GPI-specific atherosclerotic plaque-derived T-cell clones produced IFN- $\gamma$ . Values are mean $\pm$ Standard Deviation (SD) number of SFC per  $10^5$  cultured cells over background levels. Numbers of IL-17 SFC after stimulation of atherosclerotic plaque derived T-cell clones with medium alone, or  $\beta$ 2GPI (B). T-cell blasts from each clone were stimulated for 48 h with medium alone ( $\square$ ), or  $\beta$ 2GPI ( $\blacksquare$ ) in the presence of irradiated autologous antigen-presenting cells in ELISPOT microplates coated with anti-IL-17 antibody. IL-17 SFC were then counted by using an automated reader. After specific stimulation 37 of 71  $\beta$ 2GPI-specific atherosclerotic plaque-derived T-cell clones produced IL-17. Values are mean $\pm$ SD number of SFC per  $10^5$  cultured cells over background levels.

clones was assessed by using antigen-pulsed  $^{51}\text{Cr}$ -labeled autologous EBV-B cells as targets. At an E:T ratio of 10:1, all Th1 and Th17/Th1 specific T-cell clones were able to lyse  $\beta$ 2GPI-presenting autologous Epstein-Barr virus (EBV)-B cells (range of specific  $^{51}\text{Cr}$  release, 35-65%), whereas autologous EBV-B cells pulsed with control ag and co-cultured with the same clones were not lysed (Figure 7A). Likewise 2 Th0 and all Th17 specific T-cell clones were able to lyse their target (specific  $^{51}\text{Cr}$  release: 50% and 25-45% respectively), while no lysis was observed when using autologous EBV-B cells pulsed with the control ag.

Fas-FasL mediated apoptosis was assessed using Fas $^+$  Jurkat cells as target. T-cell blasts from each clone were co-cultured with  $^{51}\text{Cr}$ -labeled Jurkat cells at an E:T ratio of

10, 5, and 2.5 to 1 for 18 h in the presence of PMA and ionomycin (Figure 7B). Upon mitogen activation, 27 out of 30 Th1, 24/27 Th17/Th1, 4/10 Th17, and 2 out of 4 Th0 clones were able to induce apoptosis in target cells (range of specific  $^{51}\text{Cr}$  release: 25-61%).

## Discussion

Several clinical studies and experimental models suggest a role for aPL in accelerating atherosclerotic plaque formation in SLE. On the other hand, there is growing evidence that aPL represent a risk factor for arterial thrombosis supporting their pathogenic role in cardiovascular events.<sup>1,3,4,32</sup> Here, we report for the first time that a pro-inflammatory

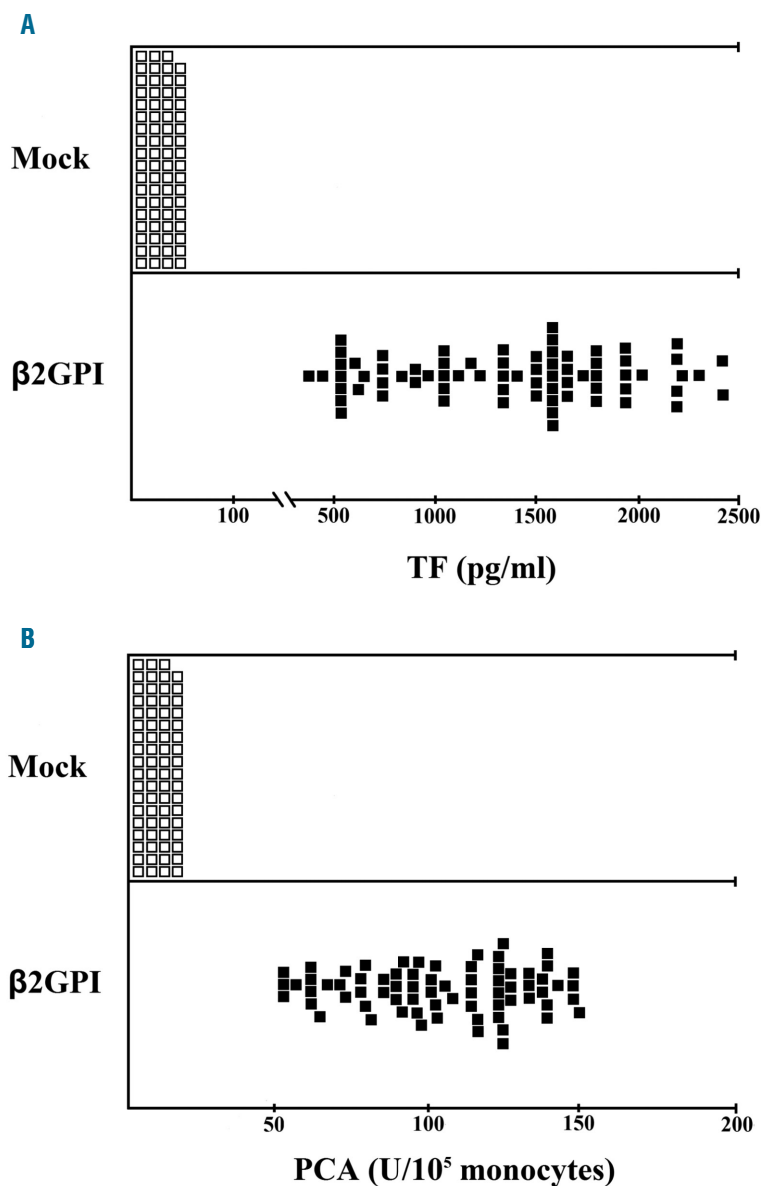
and pro-coagulant  $\beta$ 2GPI-specific Th17, Th1 and Th17/Th1 infiltrate in human atherosclerotic lesions of patients with SLE-APS and may represent a key pathogenic atherothrombotic mechanism.

Many self antigens, such as oxLDL, may theoretically be involved in SLE-APS atherosclerosis; oxLDL-specific peripheral blood-derived T cells displaying a Th1 profile were reported in APS patients.<sup>33</sup> However, there is no information on whether these cells are actively involved in atherosclerotic tissue lesions of SLE-APS patients. In addition,  $\beta$ 2GPI was found to bind ox-LDL<sup>34</sup> raising the issue of whether or not the immune response is against ox-LDL or  $\beta$ 2GPI itself.

The relevance of the data presented in this paper consists in the demonstration that all ten SLE-APS patients with clinically severe atherothrombosis harbored in their target tissues, such as atherosclerotic lesions, *in vivo*-activated CD4<sup>+</sup> T cells able to react to  $\beta$ 2GPI. CD4<sup>+</sup> T cells specific for  $\beta$ 2GPI were found also in the plaques of SLE aPL-positive patients but not in SLE aPL-negative patients

nor in atherosclerotic patients without SLE. The results suggested that  $\beta$ 2GPI drive inflammation in atherosclerotic plaques in SLE-APS and SLE aPL-positive patients, while in SLE aPL-negative patients and in non-SLE patients other antigens are involved in sustaining plaque inflammation. With the experimental procedure used in this study, the proportion of  $\beta$ 2GPI-specific CD4<sup>+</sup> T-cell clones generated from atherosclerotic plaques of atherothrombotic SLE-APS patients is remarkably higher than the frequency of  $\beta$ 2GPI-specific T cells found in their peripheral blood.

In order to investigate plaque instability, we investigated fresh T cells coming from the atherosclerotic plaques of SLE-APS patients and we found that plaque-derived CD4<sup>+</sup> T cells specifically produce IFN- $\gamma$  and IL-17 in response to both  $\beta$ 2GPI and to mitogen stimulation. Studying at clonal level the  $\beta$ 2GPI-specific T cells found in the inflammatory atherosclerotic infiltrates of SLE-APS we found that 42% were polarized T helper 1 cells, 38% were Th17/Th1 cells, 15% were polarized Th17 cells, 5% were Th0 cells, and no T cells were polarized Th2 cells. The lack of Th2 cells

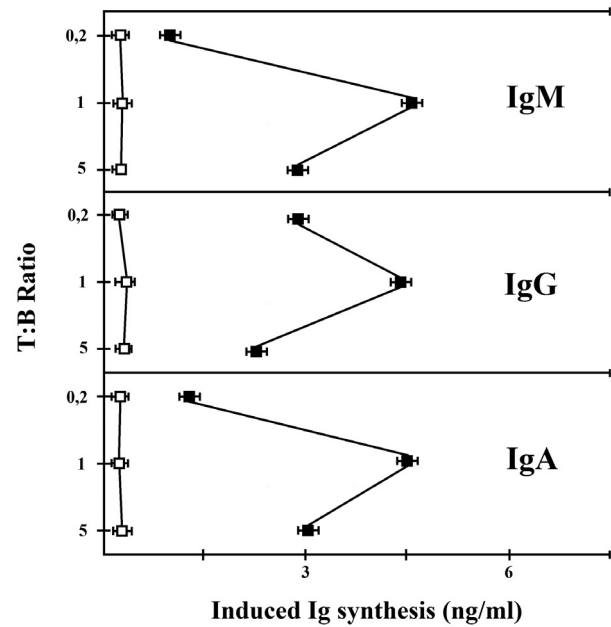


**Figure 5. Induction of tissue factor (TF) synthesis and procoagulant activity (PCA) by atherosclerotic plaque  $\beta$ 2GPI-specific T cells derived from systemic lupus erythematosus patients with antiphospholipid syndrome.** Atherosclerotic plaque  $\beta$ 2GPI-specific T cells induce TF production and PCA by autologous monocytes. To assess their ability to induce TF production and PCA by autologous monocytes,  $\beta$ 2GPI-specific Th clones were co-cultured with autologous monocytes in the presence of medium ( $\square$ ) or  $\beta$ 2GPI ( $\blacksquare$ ) (A). TF production by monocytes was assessed by ELISA. The results shown represent TF levels induced by T-cell clones over the TF production in cultures of monocytes alone. Atherosclerotic plaque-derived  $\beta$ 2GPI-specific T-cell-induced PCA in autologous monocytes (B).  $\beta$ 2GPI-specific Th clones were co-cultured with autologous monocytes in the presence of medium ( $\square$ ) or  $\beta$ 2GPI ( $\blacksquare$ ). At the end of the culture period, cells were disrupted and total PCA was quantitated as reported in the Methods section. The results shown represent PCA induced by T-cell clones in monocytes over the PCA in cultures of monocytes alone.

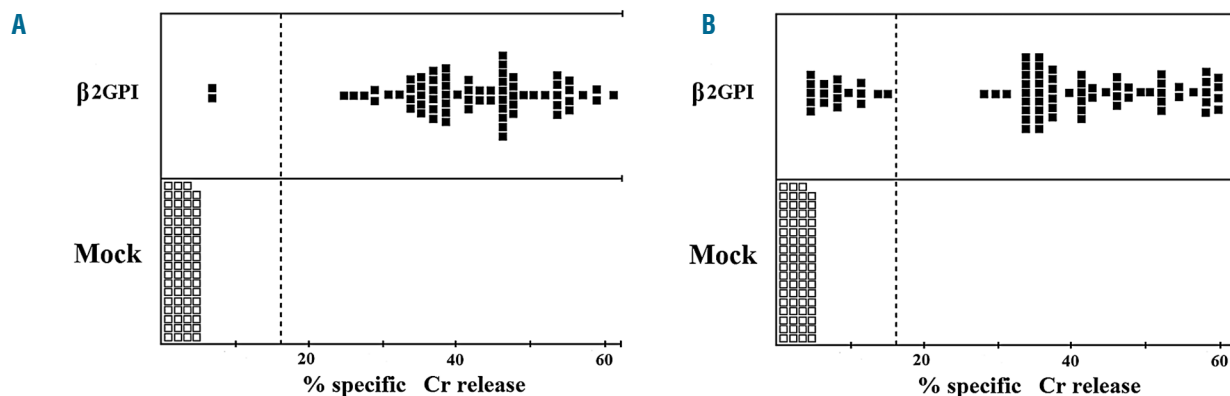
is an important risk factor in the genesis of atherosclerosis. Indeed, T cells play an important role in the genesis of atherosclerosis that has been defined a Th1-driven immunopathology,<sup>35,36</sup> and we have demonstrated that Th1 cells, producing high levels of IFN- $\gamma$ , are crucial for the development of the disease.<sup>16,20,22</sup> Given that atherosclerosis can occur and progress even in IFN- $\gamma$ - or IFN- $\gamma$ R-deficient mice, although with a lower lesion burden,<sup>37</sup> other Th cells and factors are presumably involved in the genesis of the atheroma. A third subset of effector Th cells, namely Th17, has been discovered.<sup>38</sup> Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions.<sup>39,40</sup> In the lymphocytic infiltrates of SLE-APS atherosclerotic plaques, we have found the presence of *in vivo*-activated plaque-infiltrating T cells able to produce IL-17 and IL-21 in response to  $\beta$ 2GPI. Among the clonal progeny of T cells infiltrating the lesions, we demonstrated the presence of  $\beta$ 2GPI-specific T cells able to secrete IL-17. A significant number (27%) of IL-17-producing T cells are also IFN- $\gamma$  producers. This finding is in agreement with a previous report that demonstrated the concomitant production of IL-17 and IFN- $\gamma$  by human coronary artery-infiltrating T cells in non SLE patients.<sup>41-43</sup> Plaque rupture and thrombosis are notable complications of atherosclerosis.<sup>16,43</sup> The methodology used to obtain the plaque derived T cells encompasses a clonal expansion step, followed by limiting dilution to obtain single clones. The  $\beta$ 2GPI-reactive CD4+ T-cell clone found in atherosclerotic plaques were unique, based on the T-cell receptor - V $\beta$  results obtained in the study. The  $\beta$ 2GPI-specific T-cell clones revealed their ability, not only to induce macrophage production of TF upon antigen stimulation, but that they were also able to promote PCA.

Th17 cells were shown to play a key role in experimental mouse models of atherosclerosis; IL-17 is proatherogenic in an experimental model of accelerated atherosclerosis in the presence of a high fat diet (HFD).<sup>44</sup> In fact, in IL-17<sup>-/-</sup> mice fed with HFD, the aortic lesion size and lipid

composition as well as macrophage accumulation in the plaques were significantly diminished, and the progression of the process was remarkably reduced compared with WT mice. Furthermore IL-21 was produced by almost all Th1 and Th17/Th1 cells specific for  $\beta$ 2GPI. IL-21 is actually up-regulated in patients with peripheral



**Figure 6. Helper function of atherosclerotic plaque  $\beta$ 2GPI-specific T cells derived from systemic lupus erythematosus patients with antiphospholipid syndrome.** Autologous peripheral blood B cells ( $5 \times 10^4$ ) were co-cultured with  $\beta$ 2GPI-specific T-cell blasts at a T:B ratio of 0.2, 1, and 5 to 1 in the absence (□) or presence of  $\beta$ 2GPI (■). After ten days, culture supernatants were harvested and tested for the presence of IgM, IgG, and IgA by ELISA. Results represent mean value ( $\pm$ SE) of Ig levels induced by T-cell clones compared to the Ig spontaneous production in B-cell cultures alone.



**Figure 7. Cytotoxic and pro-apoptotic activity of  $\beta$ 2GPI-specific atherosclerotic plaque-derived CD4+ T cells derived from systemic lupus erythematosus patients with antiphospholipid syndrome.** (A) To assess their cytotoxicity,  $\beta$ 2GPI-specific CD4+ T-cell clones were co-cultured at different E:T ratios with  $^{51}$ Cr-labeled autologous Epstein-Barr virus cells pulsed with  $\beta$ 2GPI (■) or medium alone (□).  $^{51}$ Cr release was measured as index of specific target cell lysis. (B) To assess their ability to induce apoptosis in target cells,  $\beta$ 2GPI-specific CD4+ T-cell clones stimulated with mitogen (■) or medium alone (□) were co-cultured with  $^{51}$ Cr-labeled Fas Jurkat cells, and  $^{51}$ Cr release was measured as the index of apoptotic target cell death.

artery diseases.<sup>45</sup> Expression of IL-17 in human atherosclerotic lesions is associated with increased inflammation and plaque vulnerability, and increased Th17 cells.<sup>46</sup> An increased incidence of atherosclerosis associated with peripheral blood Th17 responses has been demonstrated in patients with SLE.<sup>47</sup>

We have demonstrated that  $\beta$ 2GPI was able to activate Th17 and Th1 responses in atherosclerotic lesions of SLE-APS patients. The relevance of Th17/Th1 cells in non-SLE-atherosclerosis patients have been demonstrated in other studies,<sup>48,49</sup> suggesting that Th1 and Th17 cells might plastically shift into each other in different phases of the disease. It has been shown that Th17 cells might shift towards Th1 but not to Th2 *via* IL-12 receptor signaling.<sup>50</sup>

Overall, our findings support the concept that a crucial component of atherosclerosis in SLE-APS is represented by T-cell-mediated immunity and that chronic Th response against  $\beta$ 2GPI plays an important role in the genesis of atheroma in SLE-APS patients.<sup>51</sup> Among  $\beta$ 2GPI-specific IL-17-producing Th cells, the majority were polarized Th17 cells, whereas others were able to produce both

IFN- $\gamma$  and IL-17. Thus, it is possible to speculate that Th17 and Th1 cells co-migrate to the inflamed tissue and cooperate in the ongoing inflammatory process within the atherosclerotic lesion.<sup>16,17,39,52</sup>

In addition, upon appropriate Ag stimulation, the majority of atherosclerotic plaque-derived  $\beta$ 2GPI-specific clones induced both perforin-mediated cytolysis and Fas/FasL-mediated apoptosis in target cells and were able to drive the upregulation of TF production by monocytes within atherosclerotic plaques, thus further contributing to the thrombogenicity of lesions.<sup>42,43,53</sup> Our results demonstrate that  $\beta$ 2GPI is a major factor able to drive Th17 and Th1 inflammatory process in SLE-APS atherosclerosis, and suggest that Th17/Th1 cell pathway and  $\beta$ 2GPI may represent important targets for the prevention and treatment of the disease.

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