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Interleukin-17/Interleukin-21 and Interferon-γ producing T cells specific for β2 Glycoprotein I in atherosclerosis inflammation of systemic lupus erythematosus patients with antiphospholipid syndrome

Running title: Th17/Th1 inflammation in lupus atherosclerosis

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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 β2-Glycoprotein I. We investigated the cytokine production induced by β2-Glycoprotein I in activated T cells that infiltrate *in vivo* atherosclerotic lesions of lupus-antiphospholipid syndrome patients. We examined the helper function of β2-Glycoprotein I-specific T cells for the tissue factor production, as well as their cytolytic potential and their helper function for antibody production. Lupus-antiphospholipid syndrome patients harbor *in vivo* activated $CD4^+$ T cells that recognize β2-Glycoprotein I in atherosclerotic lesions. β2-Glycoprotein I induces T cell proliferation and expression of both Interleukin-17/Interleukin-21 and Interferon-γ in plaque-derived T cell clones. β2-Glycoprotein I-specific T cells display strong help for monocyte tissue factor production, and promote antibody production in autologous B cells. Moreover, plaque-derived β2-Glycoprotein I-specific $CD4^+$ T lymphocytes express both perforin-mediated and Fas/FasLigand-mediated-cytotoxicity. Altogether, our results indicate that β2-Glycoprotein I is able to elicit a local Interleukin-17/Interleukin-21 and Interferon-γ 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.

Keywords

β2-glycoprotein I, T cell, interferon-gamma, interleukin-17, interleukin-21, tissue factor

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that can be 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 β2-glycoprotein I (β2GPI)[1]. Besides the role in the acquired pro-coagulant diathesis, aPL have been also associated to accelerated atherosclerosis to explain cardiovascular manifestations of the syndrome[2-4]. An accelerated atherosclerosis in SLE has been initially demonstrated in 1975 by Bulkley et al.[5], in a necroscopic study, that was further confirmed by Urowitz et al.[6].

Many studies highlighted that SLE is associated with coronary heart disease and aterosclerosis[7-9]; an important prospective study have demonstrated that SLE patients have an accelerated progression of carotid plaque formations compare to non lupus controls[10]. SLE patients have a reduced life expectancy mainly due to increased prevalence of cardiovascular diseases. The major cardiovascular events are 2.5 higher in SLE patients compared to the general population. SLE women, age range 35-44, in respect to healthy subjects have a 50 times increased risk of myocardial infarction and accelerated atherosclerosis, that is a well recognized comorbidity in SLE[11,12].

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 the attention has been focused on inflammatory processes[13,14]. Observations in humans and animals suggest that atherosclerotic plaques derive from a 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 remodelling of the arterial wall, eventually favouring plaque instability and rupture[15]. Within the T cell population

infiltrating the plaque, most cells are activated $CD4^+$ T helper (Th) 1 and Th17 cells expressing HLA-DR and the interleukin (IL)-2 receptor (CD25)[16,17].

Current evidence indicates that autoimmunity can be detected within the atherosclerotic lesions[18]. Accordingly, self-phospholipids, such as oxidized low-density lipoprotein (oxLDL) and human heat shock proteins drive T cell inflammation in atherosclerotic patients[19,20]. 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-β2GPI-specific response in the target organ may contribute to atherothrombosis in SLE-APS patients. This hypothesis is largely based on the β2GPI presence in human atherosclerotic plaques[21,22] and on the enhanced fatty streak formation in transgenic atherosclerosis-prone mice immunized with β2GPI[23,24]. Moreover, β2GPI-reactive T cells have also been found to promote early atherosclerosis in LDL receptor deficient mice[25].

In this study we demonstrate that in SLE-APS patients, both IL-17 and IFN-γ are secreted by atherosclerotic plaques infiltrating Th cells in response to β2GPI and suggest that β2GPI 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 Data file.*

Reagents

Human β2GPI was purified as described[26]. We ruled out the presence of contaminants by a limulus test. The human β2GPI used has been tested with a limulus test and resulted negative throughout the whole study.

Patients

Upon approval of the local Ethical Committee, 10 patients (10 females, mean age 51; range 42-56 years) with SLE-APS, 10 aPL negative patients (10 females, mean age 51, range 43-55), 5 SLE aPL-positive patients (5 females, mean age 49, range 44-53), and 5 SLE aPL-negative patients (5 females, mean age 50, range 44-56), all affected by carotid atherosclerotic arteriopathy were included in the study. The carotid plaques were obtained by endoarterectomy from each patient. The clinical informations of each patient are reported in Table S1, S2, S3, and 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 APS, although they were positive for aPL, with serum anti-β2GPI, 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-β2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant.

Anti-phospholipid antibody detection

The detection of aCL and aβ2GPI in patient sera, and analysis of LA was performed as described elsewhere[28],[29].

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[16]. 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 Vβ chain of β2GPI-specific Th clones was analyzed with a panel of mAbs specific to the following: Vβ1, Vβ2, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2 and Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, and Vβ23 (Beckman Coulter); Vβ6.7 (Gentaur) and Vβ3.1 (In Vitro Gen). Isotype-matched nonspecific Ig were used as negative control. Vβ10, Vβ15, and Vβ19 T cell receptor typing were investigated by Clontech kit, according to the manufacturer's instructions. Each β2GPI-reactive $CD4^+$ T cell clone was stained by only one of the TCR-Vβ chain–specific monoclonal antibodies, showing a single peak of fluorescence intensity (Fig. S1) . The cytokine production, the cytotoxicity, the helper functions for antibody and tissue factor production of β2GPI-specific T cell clones were performed as described[16, 30, 31].

Statistical analysis

Statistical analyses were performed using Student's *t* test; data were considered significant if p values < 0.05.

Results

Atherosclerotic lesions of SLE-APS patients and SLE aPL-positive patients harbour autoreactive β2GPI-specific $CD4^+$ 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 $CD4^+$ and 37 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of 10 SLE-APS patients. For each patient, $CD4^+$ and $CD8^+$ atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium, or β2GPI. None of the $CD8^+$ T cell clones showed proliferation to β2GPI although they proliferated in response to mitogen stimulation (Fig. 1). We have also investigated the amount of β2GPI-specific T cells present in the peripheral blood of SLE-APS patients and compared it with the one found in atheromas. The proportion of β2GPI-specific $CD4^+$ T cell clones generated from atherosclerotic plaques of SLE-APS patients was 24%, which is remarkably higher compared with the frequency of β2GPI-specific T cells found in the peripheral blood of the same patients (between 1:1900 and 1:3400).

Seventy-one (24%) of the 297 $CD4^+$ T cell clones generated from SLE-APS atherosclerotic plaque-infiltrating T cells proliferated significantly to β2GPI (Fig. 1). Each SLE-APS patient displayed a comparable percentage of $CD4^+$ T cell clones responsive to β2GPI (Table S1). On the other hand, a total number of 288 $CD4^+$ and 42 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of 10 atherotrombotic patients, that were negative for aPL. For each patient, $CD4^+$ and $CD8^+$ atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium or β2GPI. None of the $CD4^+$ or $CD8^+$ T cell clones derived from the atherosclerotic lesions showed proliferation to β2GPI (Table S2). A total number of 135 $CD4^+$ and 21 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of 5 SLE aPL-positive. For each

patient, $CD4^+$ and $CD8^+$ atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium or β2GPI. 25 $CD4^+$ and no $CD8^+$ T cell clones derived from the atherosclerotic lesions of SLE aPL-positive patients showed proliferation to β2GPI (Table S3). A total number of 136 $CD4^+$ and 30 $CD8^+$ T cell clones were obtained from atherosclerotic lesions of 5 SLE aPL-negative. For each patient, $CD4^+$ and $CD8^+$ atherosclerotic lesion-derived T cell clones were assayed for proliferation in response to medium or β2GPI. None of the $CD4^+$ or $CD8^+$ T cell clones derived from the atherosclerotic lesions showed proliferation to β2GPI (Table S4).

All β2GPI-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 β2GPI and autologous APCs. Then, TNF-α and IL-4, IFN-γ and IL-17 production was measured in culture supernatants. Upon antigen stimulation with β2GPI of the 71 β2GPI-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-α (Th0 clones) (Fig. 2). Upon antigen stimulation with β2GPI of the 25 β2GPI-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 1 was Th0 (Fig. 3). T cell blasts from each of the 71 β2GPI-reactive T cell clones obtained from atherosclerotic lesions of patients with SLE-APS were further screened by IFN-γ and IL-17 ELISPOT in response to β2GPI. Upon appropriate stimulation, 61 atherosclerotic-derived $CD4^+$ T cell clones produced IFN-γ, and thirty-seven produced IL-17 (Fig. 4). Interestingly, all IL-17-producing β2GPI-reactive T cell clones, produce IL-21 (mean ± SE, 3.3 ± 0.5 ng/mL per 10^6 T cells) in response to antigen stimulation.

β2GPI-specific atherosclerotic lesion-infiltrating T cells help monocyte TF production and PCA

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 β2GPI-specific T cells had the potential to express helper functions for TF production and PCA by autologous monocytes. Antigen-stimulated β2GPI-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 (Fig. 5A) production and PCA (Fig. 5B) by autologous monocytes.

Atherosclerotic lesion-derived β2GPI-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 β2GPI-specific T cell clones to provide B cell help for Ig synthesis was 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 β2GPI and at a T-to-B cell ratio of 0.2 to 1, all of the β2GPI-specific T cell clones provided substantial help for Ig production. At a 1-to-1 T/B cell ratio, β2GPI-dependent T cell help for IgM, IgG, and IgA production by B cells was remarkably higher (Fig. 6). However, at a 5-to-1 T/B cell ratio, co-culturing B cells with autologous β2GPI-specific T cell clones in the presence of β2GPI resulted in a much lower Ig synthesis.

Atherosclerotic lesion-derived β2GPI-specific T cell clones display cytotoxic and pro-apoptotic activity.

The cytolytic potential of SLE-APS-derived atherosclerotic lesion-derived β2GPI-specific autoreactive T cell clones was assessed by using antigen-pulsed ^{51}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 β2GPI-presenting autologous EBV-B cells (range of specific ^{51}Cr release, 35–65%), whereas autologous EBV-B cells pulsed with control ag and co-cultured with the same clones were not lysed (Fig. 7A). Likewise 2 Th0 and all Th17 specific T cell clones were able to lyse their target (specific ^{51}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}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 (Fig. 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}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[1,3,4,32]. Here in we report for the first time that a pro-inflammatory and pro-coagulant β2GPI-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[33]. However, there is no information on whether these cells are actively involved in atherosclerotic tissue lesions of SLE-APS patients. In addition, β2GPI was found to bind ox-LDL[34] raising the issue whether or not the immune response is against ox-LDL or β2GPI 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 $CD4^+$ T cells able to react to β2GPI. $CD4^+$ T cells specific for β2GPI 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 β2GPI 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 β2GPI-specific $CD4^+$ T cell clones generated from atherosclerotic plaques of atherotrombotic SLE-APS patients is remarkably higher than the frequency of β2GPI-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 $CD4^+$ T cells specific produce IFN-γ and IL-17 in response to both β2GPI and to mitogen stimulation. Studying at clonal level the β2GPI-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 none of T cells were polarized Th2 cells. The lack of Th2 cells is an important risk factor in the genesis of atherosclerosis. T cells indeed play an important role in the genesis of atherosclerosis that has been defined a Th1-driven immunopathology[35,36], and we have demonstrated that Th1 cells, producing high levels of IFN-γ, are crucial for the development of the disease[16,20,22]. Given that atherosclerosis can occur and progress even in IFN-γ- or IFN-γR–deficient mice, although with a lower lesion burden[37], 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[38]. Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions[39,40]. 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 β2GPI. Among the clonal progeny of T cells infiltrating the lesions, we demonstrated the presence of β2GPI -specific T cells able to secrete IL-17. A significant number (27%) of IL-17–producing T cells are also IFN-γ producers. This finding is in agreement with a previous report that demonstrated the concomitant production of IL-17 and IFN-γ by human coronary artery-infiltrating T cells in non SLE patients[41-43]. Plaque rupture and thrombosis are notable complications of atherosclerosis[16,43]. The methodology used to obtain the plaque derived T-cells encompasses a clonal expansion step, followed by limiting dilution to obtain single clones. The β2GPI-reactive $CD4^+$ T cell clone found in atherosclerotic plaques were unique, based on the T-cell receptor - Vβ results obtained in the study. The β2GPI -specific T-cell clones revealed their

ability not only to induce macrophage production of TF upon antigen stimulation but also were able to promote PCA.

Th17 cells were shown to play a key role in experimental mouse models of atherosclerosis; IL-17 is proatherogenic in experimental model of accelerated atherosclerosis in the presence of high fat diet (HFD)[44]. In fact, in IL-17−/− 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 β2GPI. IL-21 is actually up-regulated in patients with peripheral artery diseases[45]. Expression of IL-17 in human atherosclerotic lesions is associated with increased inflammation and plaque vulnerability and increased Th17 cells[46]. An increased incidence of atherosclerosis associated with peripheral blood Th17 responses has been demonstrated in patients with SLE[47].

We have demonstrated that β2GPI 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[48,49], suggesting that Th1 and Th17 cells might plastically shift one into the 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 signalling[50].

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 β2GPI plays an important role in the genesis of atheroma in SLE-APS patients[51]. Among β2GPI-specific IL-17–producing Th cells, the majority were polarized Th17 cells, whereas others were able to produce both IFN-γ 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[16,17,39,52].

In addition, upon appropriate Ag stimulation, the majority of atherosclerotic plaque-derived β2GPI-specific clones induced both perforin-mediated cytolysis and Fas/FasL-mediated apoptosis in target cells and were able to drive the up-regulation of TF production by monocytes within atherosclerotic plaques, thus further contributing to the thrombogenicity of lesions[42,43,53]. Our results demonstrate that β2GPI is a major factor able to drive Th17 and Th1 inflammatory process in SLE-APS atherosclerosis and suggest that Th17/Th1 cell pathway and β2GPI may represent important targets for the prevention and treatment of the disease.

Contribution

MB, PLM, and MMDE designed the study. MB, NC, JR, GE, AT, HST, ES, LE, JB, DP, CTB, PLM, and MMDE participated in patient data collection. All authors analyzed and interpreted the data. CDB was the methodologist of the study and AG was the study biostatistician responsible for the statistical analyses. All authors participated in writing of the report, agreed on the content of the manuscript, reviewed drafts, and approved the final version.

Disclosures

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest. MM is an employee of Inova Diagnostics Inc. PLM has taken part in speakers' bureaux for INOVA Diagnostics Inc.

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Figure legends

Figure 1. Antigen specificity of atherosclerotic plaque $CD4^+$ T and $CD8^+$ T cell clones obtained from SLE-APS patients. Both $CD4^+$ T and $CD8^+$ T cell clones were tested for antigen-specificity. T cell clones were analyzed for their responsiveness to β2GPI (10 nM) (■), or medium (□) by measuring [^{3}H]thymidine uptake after 60 h of co-culture with irradiated autologous PBMCs. 71 out of 297 $CD4^+$ T cell clones proliferated in response to β2GPI and are shown in panel **A**. None of the 37 $CD8^+$ T cell clone proliferated to β2GPI (panel **B**).

Figure 2. Cytokine profile of atherosclerotic plaque β2GPI-specific $CD4^+$ T cell clones obtained from SLE-APS patients. Th clones were tested for cytokine production (**A**-**B**). β2GPI-specific Th clones were stimulated with β2GPI and TNF-α and IL-4, IFN-γ and IL-17 production was measured in culture supernatants. In unstimulated cultures, levels of TNF-α, IL-4, IFN-γ and IL-17 were consistently < 20 pg/ml. $CD4^+$ T cell clones producing IFN-γ, but not IL-17 nor IL-4 were coded as Th1. $CD4^+$ T cell clones producing IL-17, but not IFN-γ nor IL-4 were coded as Th17. $CD4^+$ T cell clones producing IFN-γ, and IL-17, but not IL-4 were coded as Th17/Th1. $CD4^+$ T cell clones producing TNF-α and IL-4, but not IL-17 were coded as Th0.

Figure 3. Cytokine profile of atherosclerotic plaque β2GPI-specific $CD4^+$ T cell clones obtained from SLE-aPL-positive patients. Th clones were tested for cytokine production (**A**-**B**). β2GPI-specific Th clones were stimulated with β2GPI and TNF-α and IL-4, IFN-γ and IL-17 production was measured in culture supernatants. In unstimulated cultures, levels of TNF-α, IL-4, IFN-γ and IL-17 were consistently < 20 pg/ml. $CD4^+$ T cell clones producing IFN-γ, but not IL-17 nor IL-4 were coded as Th1. $CD4^+$ T cell clones producing IL-17, but not IFN-γ nor IL-4 were

coded as Th17. $CD4^+$ T cell clones producing IFN-γ, and IL-17, but not IL-4 were coded as Th17/Th1. $CD4^+$ T cell clones producing TNF-α and IL-4, but not IL-17 were coded as Th0.

Figure 4. β2GPI driven IFN-γ and IL-17 secretion by β2GPI-specific atherosclerotic plaque derived Th clones from SLE-APS patients. Numbers of IFN-γ spot-forming cells (SFCs) after stimulation of atherosclerotic plaque derived T cell clones with medium alone, or β2GPI **(A)**. T cell blasts from each clone were stimulated for 48 h with medium alone (□), or β2GPI (■), in the presence of irradiated autologous APCs in ELISPOT microplates coated with anti-IFN-γ antibody. IFN-γ SFCs were then counted by using an automated reader. After specific stimulation, 61/71 β2GPI-specific atherosclerotic plaque-derived T cell clones produced IFN-γ. Values are the mean ± SD number of SFCs per 10^5 cultured cells over background levels.

Numbers of IL-17 spot-forming cells SFCs after stimulation of atherosclerotic plaque derived T cell clones with medium alone, or β2GPI **(B)**. T cell blasts from each clone were stimulated for 48 h with medium alone (□), or β2GPI (■) in the presence of irradiated autologous APCs in ELISPOT microplates coated with anti-IL-17 antibody. IL-17 SFCs were then counted by using an automated reader. After specific stimulation 37/71 β2GPI-specific atherosclerotic plaque-derived T cell clones produced IL-17. Values are the mean ± SD number of SFCs per 10^5 cultured cells over background levels.

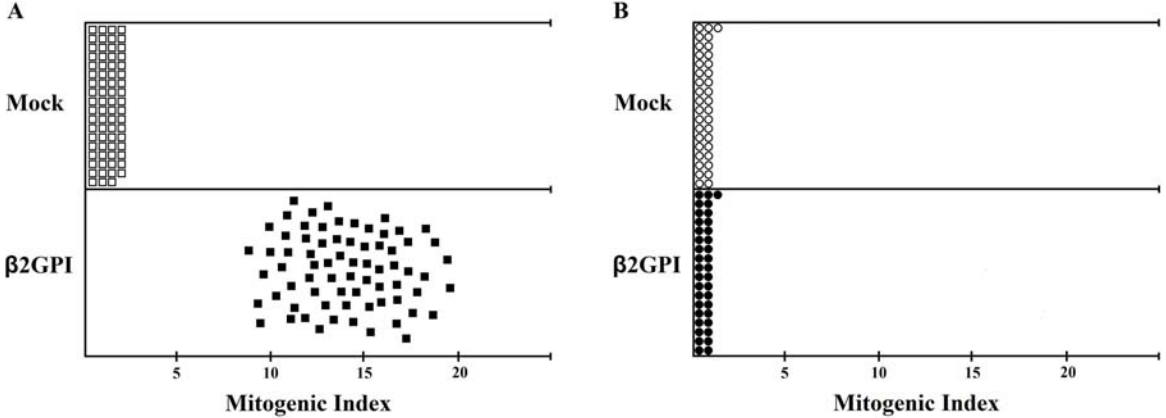
Figure 5. Induction of TF synthesis and PCA by atherosclerotic plaque β2GPI-specific T cells derived from SLE-APS patients. Atherosclerotic plaque β2GPI-specific T cells induce TF production and PCA by autologous monocytes. To assess their ability to induce TF production and PCA by autologous monocytes, β2GPI-specific Th clones were co-cultured with autologous

monocytes in the presence of medium (□) or β2GPI (■) (**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 β2GPI-specific T cell induced PCA in autologous monocytes (**B**). β2GPI-specific Th clones were co-cultured with autologous monocytes in the presence of medium (□) or β2GPI (■). At the end of the culture period, cells were disrupted and total PCA was quantitated as reported in Materials and Methods. The results shown represent PCA induced by T cell clones in monocytes over the PCA in cultures of monocytes alone.

Figure 6. Helper function of atherosclerotic plaque β2GPI-specific T cells derived from SLE-APS patients. Autologous peripheral blood B cells (5×10^4) were co-cultured with β2GPI-specific T cell blasts at a T:B ratio of 0.2, 1, and 5 to 1 in the absence (□) or presence of β2GPI (■). After 10 days, culture supernatants were harvested and tested for the presence of IgM, IgG, and IgA by ELISA. Results represent the mean value (+/–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 β2GPI-specific atherosclerotic plaque-derived $CD4^+$ T cells derived from SLE-APS patients. (**A**) To assess their cytotoxicity, β2GPI-specific $CD4^+$ T cell clones were co-cultured at different E:T ratios with ^{51}Cr-labeled autologous EBV-B cells pulsed with β2GPI (■) 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, β2GPI-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.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10
15
20
Mitogenic Index
B
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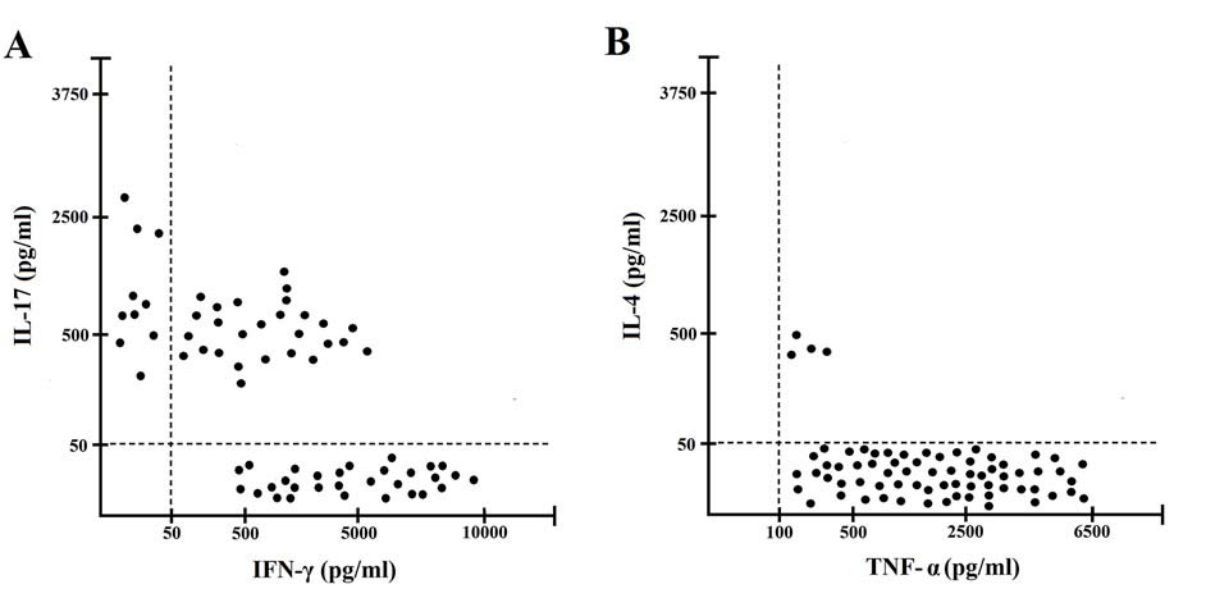
β2GPI
5
10
15
20
Mitogenic Index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500
5000
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IFN-γ (pg/ml)
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500
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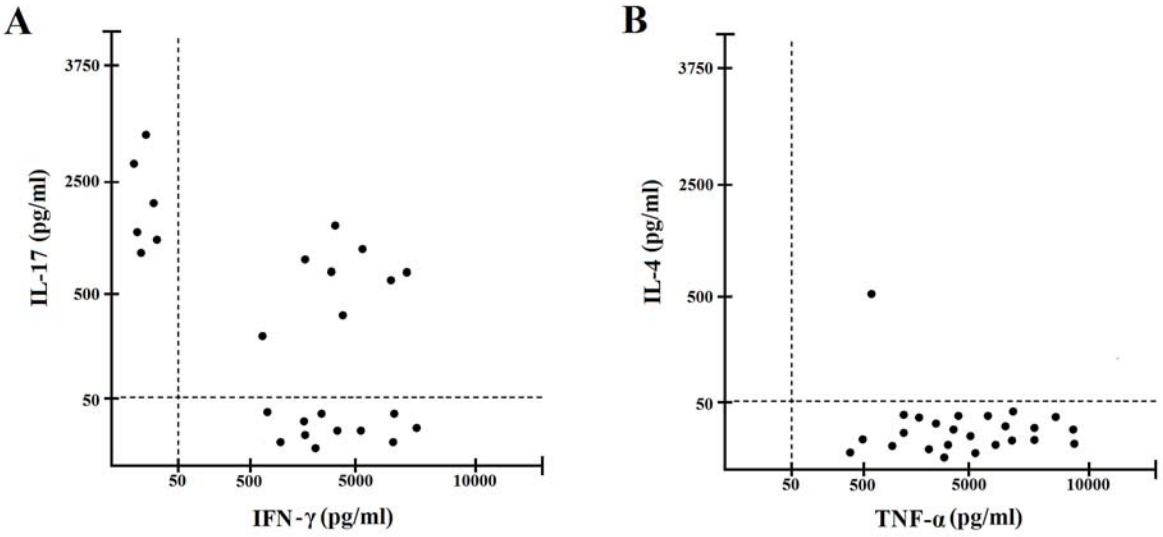
IL-4 (pg/ml)
100
500
2500
6500
TNF- α (pg/ml)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IL-4 (pg/ml)
50
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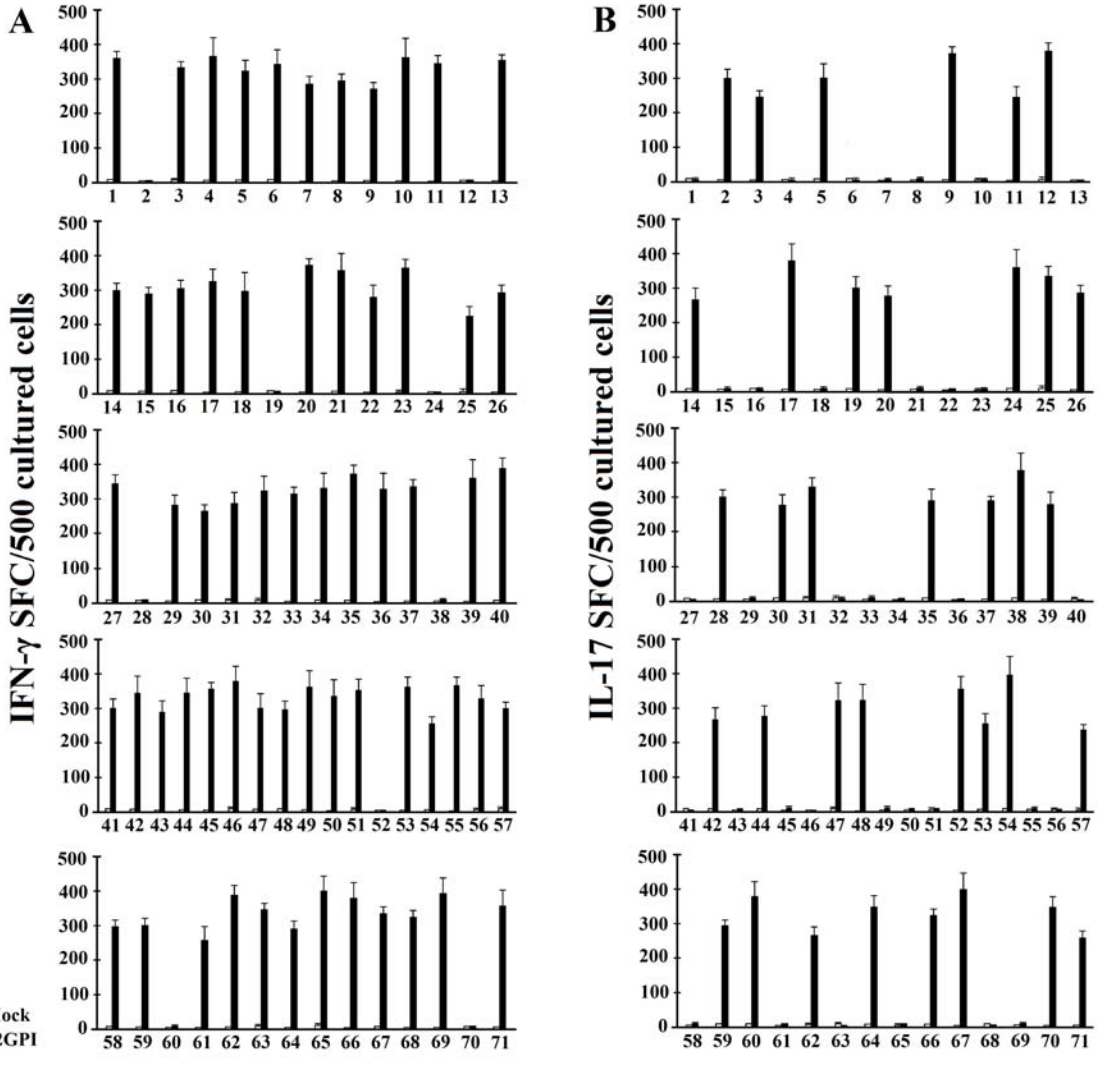
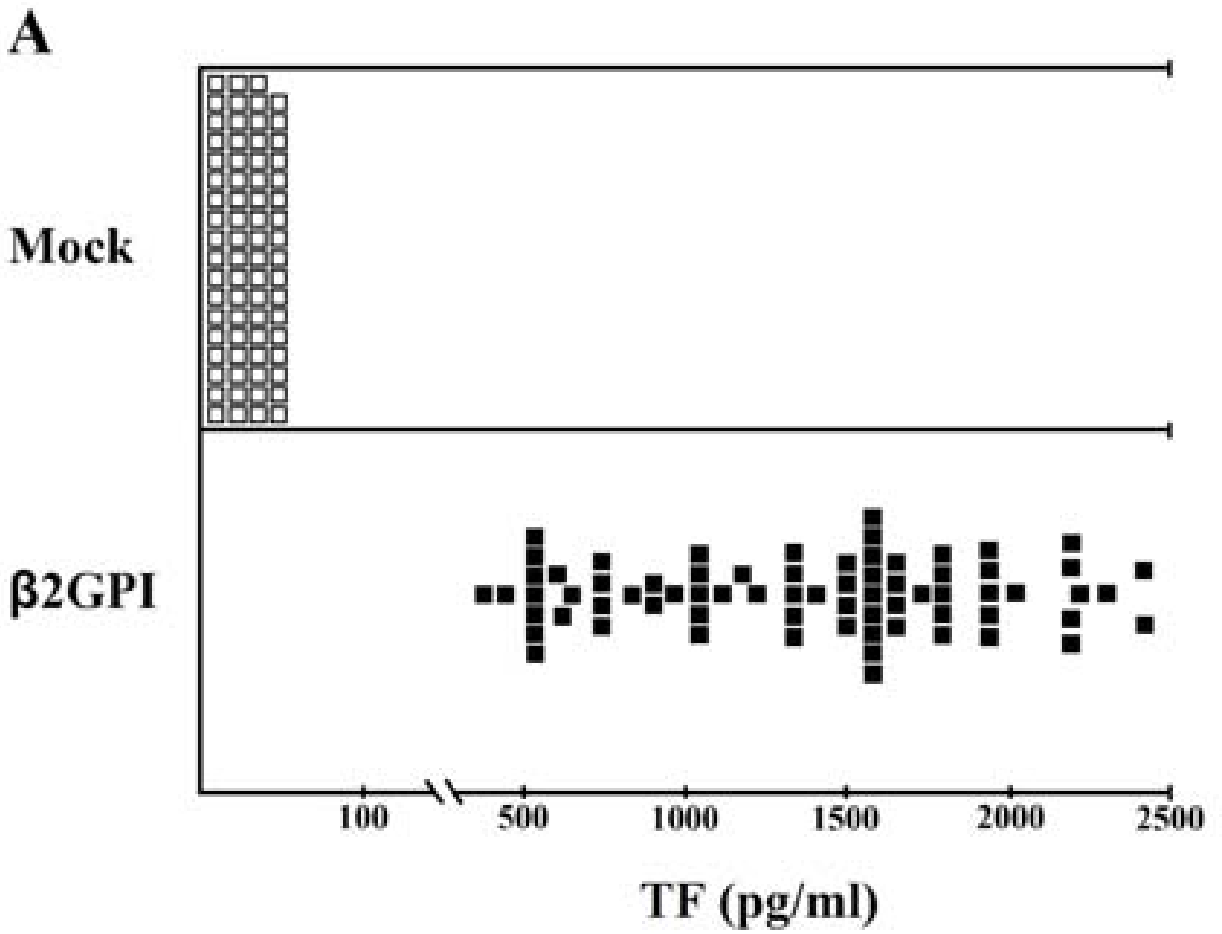
5000
10000
TNF-α (pg/ml)


A
B
IFN-γ SFC/500 cultured cells
IL-17 SFC/500 cultured cells
0
100
200
300
400
500
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 40
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57
58 59 60 61 62 63 64 65 66 67 68 69 70 71
Mock
β2GPI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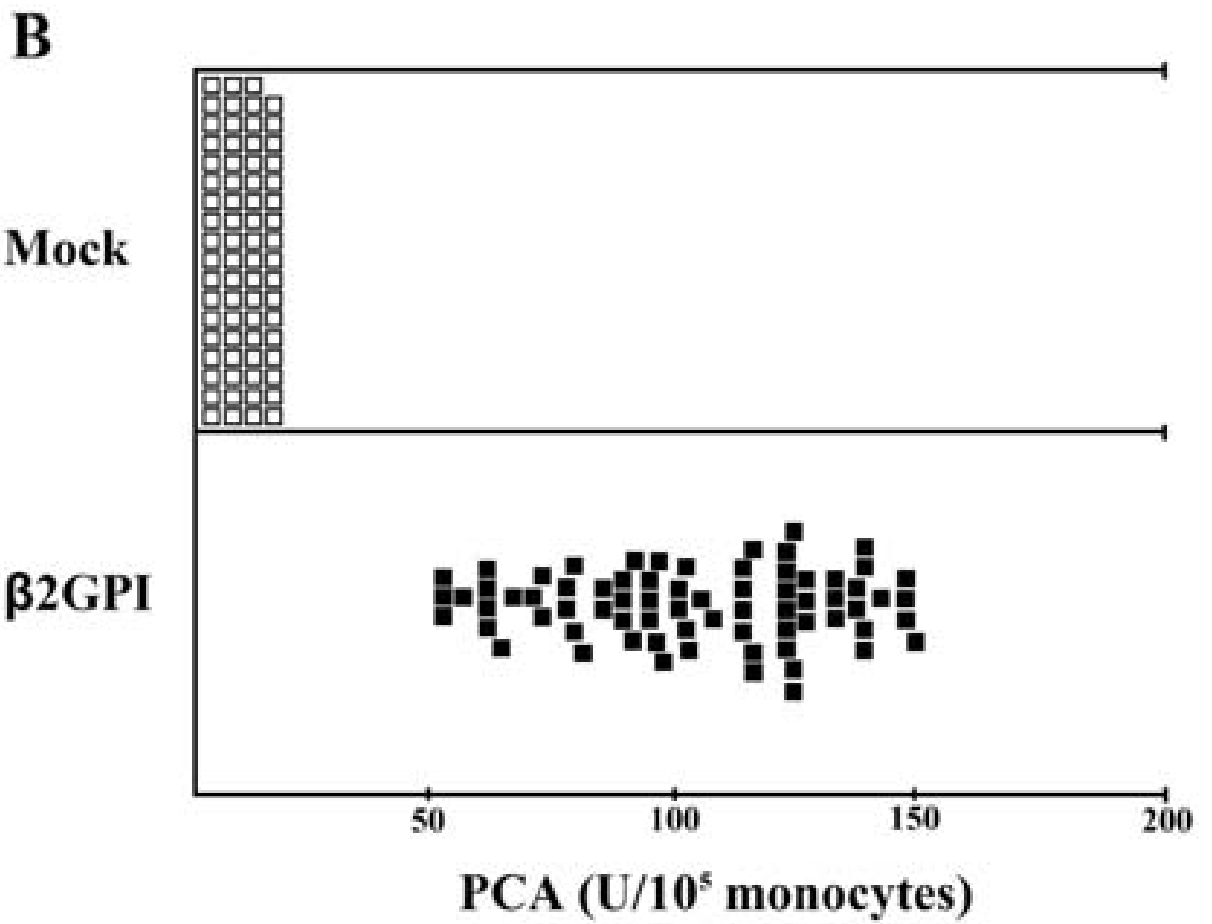
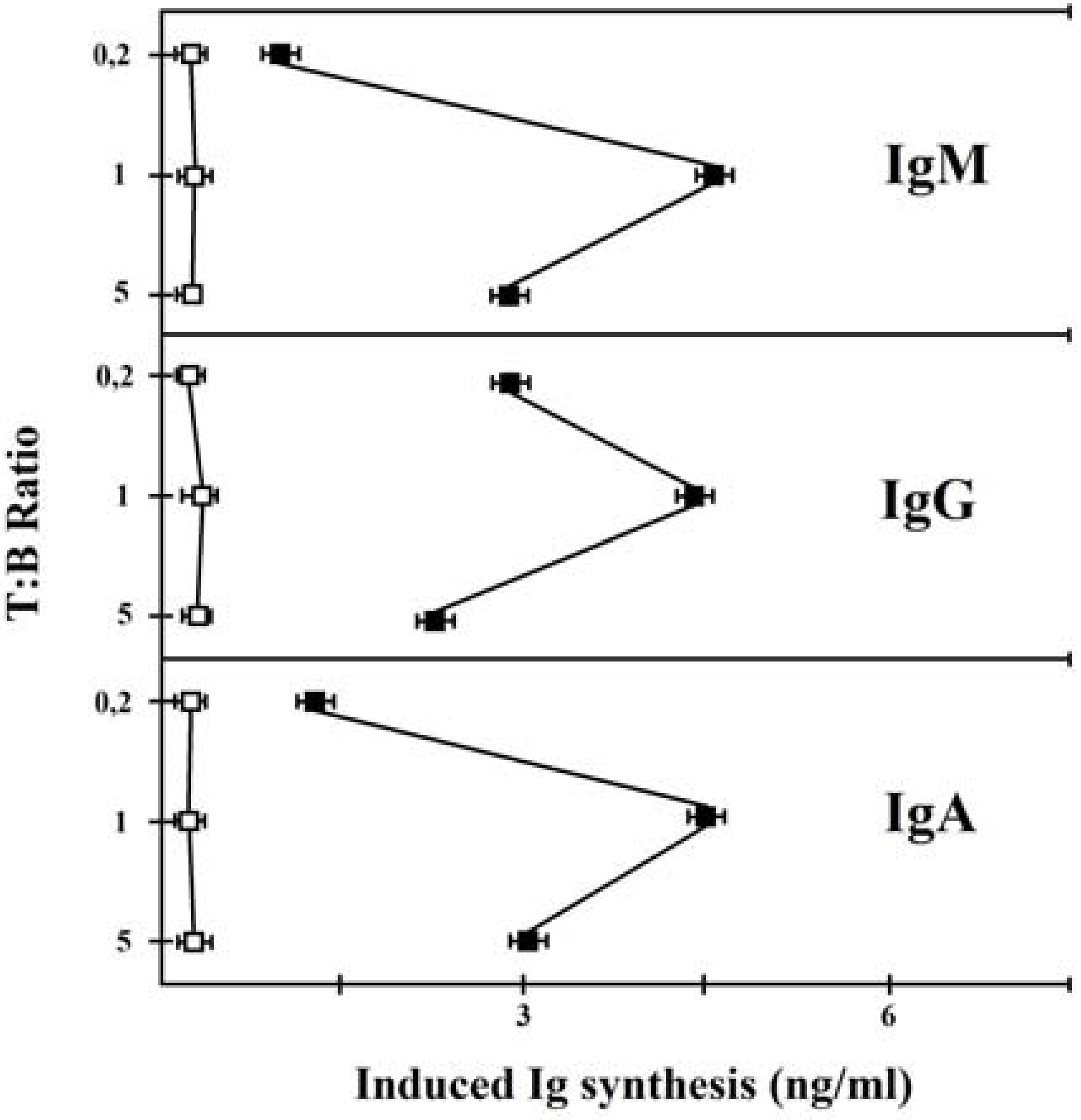
Mock
β2GPI
100
500
1000
1500
2000
2500
TF (pg/ml)


B
Mock
β2GPI
50
100
150
200
PCA (U/10⁵ monocytes)


IgM
IgG
IgA
0,2
1
5
T:B Ratio
3
6
Induced Ig synthesis (ng/ml)

Supplemental Methods

Reagents

Human β2GPI was purified by perchloric acid treatment of pooled normal human sera obtained from blood donors followed by affinity purification on Heparin column (HiTrap Heparin HP, GE Healthcare, Milan, Italy) and by ion-exchange chromatography (Resource-S, GE Healthcare)[1]. We also ruled out the presence of contaminants by a limulus test. The human β2GPI used has been tested with a limulus test and resulted negative throughout the whole study. Human recombinant (hr) interleukin (IL)-2 and tetanus toxoid (TT) were provided by Novartis, Siena, Italy. PHA was purchased from Life Technologies (Carlsbad, CA). Fluorochrome-conjugated human monoclonal antibodies (mAbs) anti-CD3, anti-CD4, anti-CD8, anti-IFNγ and isotype-matched control mAb were purchased from BD Biosciences (San Jose, CA, USA). The fluorochrome-conjugated anti-IL-17 mAb was obtained from eBioscience (San Diego, CA, USA). PMA, ionomycin and brefeldin A were purchased from BD Biosciences (San Jose, CA, USA).

Patients

Upon approval of the local Ethical Committee, 10 patients (10 females, mean age 51; range 42-56 years) with SLE-APS, 10 aPL negative patients (10 females, mean age 51, range 43-55), 5 SLE aPL-positive patients (5 females, mean age 49, range 44-53), and 5 SLE aPL-negative patients (5 females, mean age 50, range 44-56), all affected by carotid atherosclerotic arteriopathy were included in the study. The carotid plaques were obtained by endoarterectomy from each patient. The clinical informations of each patient are reported in Table S1, S2, S3, and S4.

All patients studied (SLE-APS, SLE aPL-positive, SLE aPL-negative and aPL negative patients) were eligible for vascular surgery. SLE-APS patients were triple positive for aPL, with high titers serum anti-β2GPI, anti-cardiolipin (aCL) antibodies and with positivity for Lupus Anticoagulant (LA). All SLE-APS patients in this study satisfied the Myiakis's criteria for APS, and they were on oral anticoagulation with vitamin K antagonists, then switched to low molecular weight heparin few days before surgery[2]. None of them displayed traditional risk factors for atherosclerosis and they were not receiving any anti-lipidemic drugs. All the SLE aPL-positive patients were affected by SLE but not by APS, although they were positive for aPL, with serum anti-β2GPI, 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-β2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant.

Anti-phospholipid antibody detection

For the detection of aCL and aβ2GPI in patient sera, commercially available solid-phase ELISA employing purified human β2GPI in complex with CL and human β2GPI were used (Inova, Ca, USA). Sera were considered positive when their concentration exceeded the cut-off of 10 U/mL for IgG and IgM. All samples were tested by the respective in-house assay as described elsewhere[3]. The results of the two techniques were comparable.

Analysis of LA was performed in accordance with the international recommendations as described recently[4].

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. Plaque fragments were cultured for 7 days in RPMI 1640 medium supplemented with IL-2 (50 units/ml) to expand *in vivo*-activated T cells. Specimens were then disrupted, and single T cells were cloned under limiting dilution, as described[5]. 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 Vβ chain of β2GPI-specific Th clones was analyzed with a panel of mAbs specific to the following: Vβ1, Vβ2, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2 and Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, and Vβ23 (Beckman Coulter); Vβ6.7 (Gentaur) and Vβ3.1 (In Vitro Gen). Isotype-matched nonspecific Ig were used as negative control. Vβ10, Vβ15, and Vβ19 T cell receptor typing were investigated by Clontech kit, according to the manufacturer's instructions. Each β2GPI-reactive $CD4^+$ T cell clone was stained by only one of the TCR-Vβ chain–specific monoclonal antibodies, showing a single peak of fluorescence intensity (Fig. S1) .

T cell clones were then analyzed for their responsiveness to β2GPI by measuring [^{3}H]thymidine uptake after 60 h of co-culture with irradiated autologous PBMCs in the presence of medium, or β2GPI (10 nM). The mitogenic index (MI) was calculated as the ratio between mean values of counts per minute (cpm) obtained in stimulated cultures and those obtained in the presence of medium alone. MI >5 was considered as positive.

Assessment of T cell clones cytokine profile.

To assess the cytokine production of β2GPI-specific T cell clones upon antigen stimulation, 5×10^5 T cell blasts of each clone were co-cultured for 48 h in 0.5 ml of serum-free medium with 5×10^5 irradiated autologous PBMCs in the absence or presence of β2GPI (10 nM). At the end of the

culture period, duplicate samples of each supernatant were assayed for their IFN-γ, TNF-α, IL-4, IL-21 and IL-17 (BioSource International, Camarillo, CA) production by ELISA[5]. For further investigation, T cell blasts from each β2GPI-specific T cell clone were stimulated with medium or β2GPI (10 nM) in the presence of autologous APCs for 48 h in ELISPOT microplates coated with anti-IFN-γ or anti-IL-17 antibody, respectively (eBioscience, Inc., San Diego, Ca, USA). At the end of culture period, the number of IFN-γ and IL-17 SFCs were counted as described[5].

T cell clone-mediated cytotoxicity and Fas-Fas Ligand (L) mediated proapoptotic activity.

T cell clones cytolytic activity was assessed as reported[5]. T cell blasts of β2GPI-specific T cell clones were incubated at ratios of 10, 5, and 2.5 to 1 with ^{51}Cr-labeled autologous Epstein-Barr virus transformed (EBV)-B cells pre-incubated with β2GPI (10 nM) or medium alone. After centrifugation, microplates were incubated for 8 h at 37° C, and 0.1 ml of supernatant was removed for the measurement of ^{51}Cr release, as reported[16]. The ability of β2GPI-specific T cell clones to induce Fas-FasL mediated apoptosis was assessed using Fas^{+} Jurkat cells as target. T cell blasts from each clone were co-cultured with ^{51}Cr-labeled Jurkat cells at an effector/target (E:T) ratio of 10, 5, and 2.5 to 1 for 18 h in the presence of PMA (10 ng/ml) and ionomycin (1 μmol/ml), as reported[5].

T helper assay to assess their ability to induce Tissue Factor (TF) production and procoagulant activity (PCA) in autologus monocytes.

T cell blasts (8×10^5 / ml) of β2GPI-specific T cell clones were co-cultured for 16 hrs with autologous monocytes (4×10^5 / ml) in the presence of serum-free medium or β2GPI (10 nM). At the end of the culture period, the amount of TF protein was quantitated by a specific ELISA (American

Diagnostica, Greenwich, CT) in duplicate samples of supernatants obtained from cell suspensions after solubilization of membrane proteins with Triton X-100 and ultracentrifugation, as reported[5]. At the end of culture period, cell suspensions consisting of monocytes alone, or monocytes plus activated T cells were disrupted by repeated freezing and thawing followed by sonication. Total cellular content of PCA was determined in a one-stage clotting assay and expressed in arbitrary units ($U/10^5$ monocytes) assigned by comparison with a standard curve derived from rabbit brain thromboplastin standard (Manchester Comparative Reagents, Manchester, UK), as reported[5]. Our log-log plot was linear up to 200 seconds clotting time. Values less than 10 $U/10^5$ monocytes corresponded to clotting times ranging from 170 to 80 seconds. One thousand units corresponded to approximately 22 seconds clotting time. PCA was characterized as factor VII-dependent procoagulant activity by evaluating its sensitivity to phospholipase C (Calbiochem, San Diego, CA), concanavalin A, and cysteine protease inhibitor (HgCI2), and by using factor VII- and factor X-deficient plasma samples[6].

T cell clones' helper assay to evaluate the induction of immunoglobulin (Ig) production by autologous B cell

T cell blasts of each clone were co-cultured at ratios of 0.2, 1, and 5 to 1 with autologous PBMCs in the absence or presence of β2GPI and, on day 10, IgM, IgG, and IgA levels in cell free culture supernatants were measured as previously described[7].

Statistical analysis

Statistical analyses were performed using Student's *t* test; data were considered significant if p values ≥ 0.05.

Study approval

Prior written informed consent was received from SLE patients and controls according to the Helsinki Declaration. Experiments were approved by the local Ethics Committee.

Supplemental references

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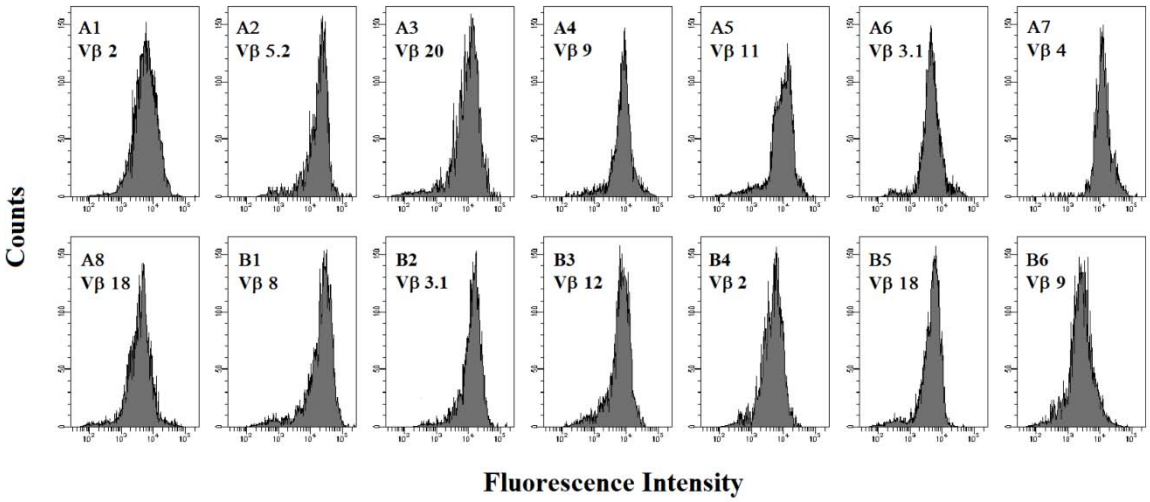
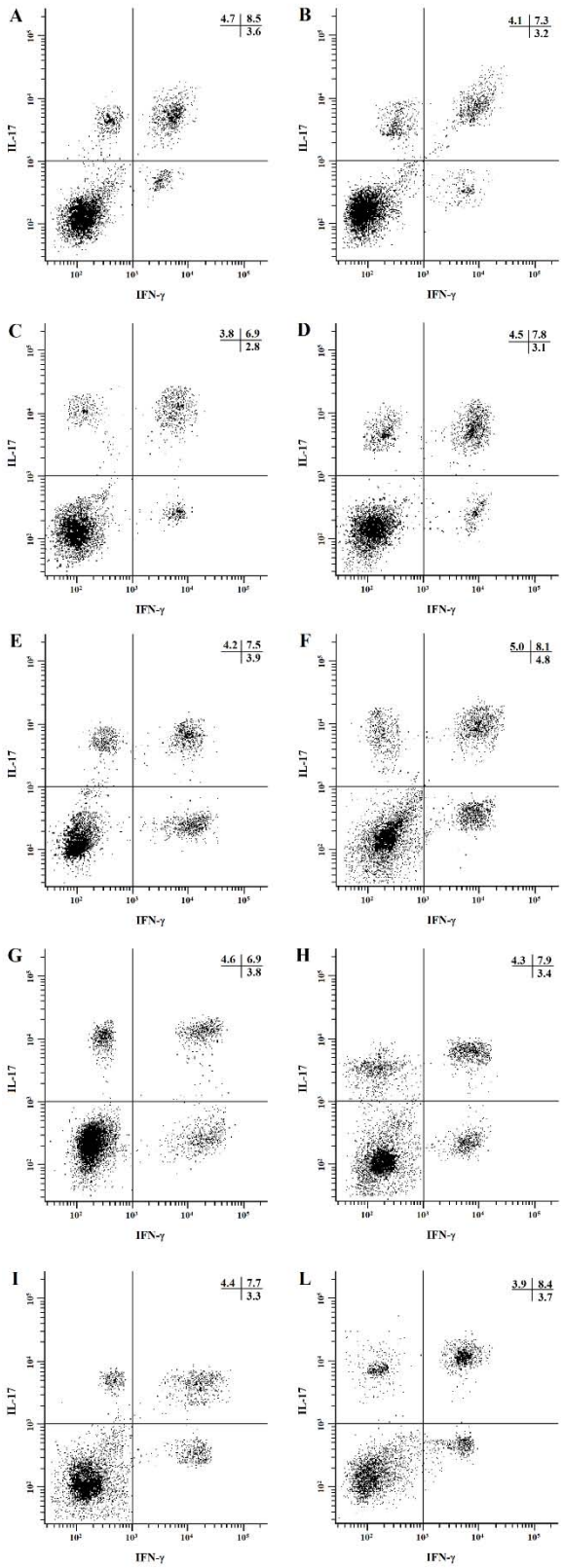
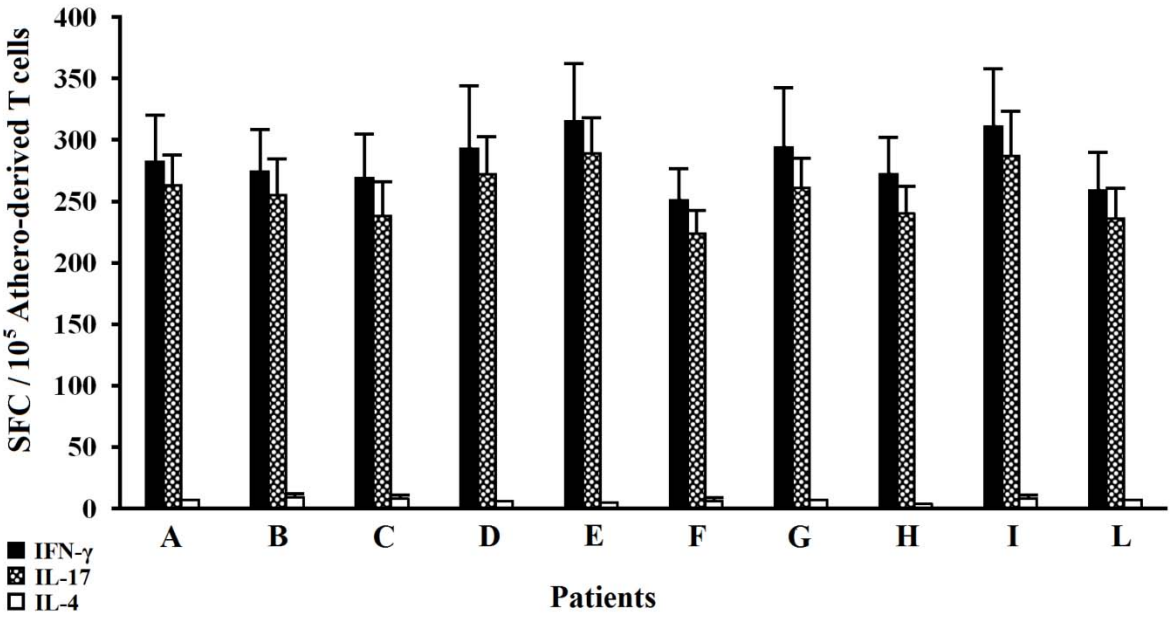
7. D'Elios MM, Bergman MP, Azzurri A, et al. $H(^+)$,$K(^+)$-atpase (proton pump) is the target autoantigen of Th1-type cytotoxic T cells in autoimmune gastritis. Gastroenterology. 2001;120(2):377–386.



Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3

Legends to Supplementary figures

Figure S1. TCR Vβ chain repertoire of β2GPI-specific T cell clones derived from the atherosclerotique plaques of SLE-APS patients. The cloality of T cell clones specific for β2GPI was analysed by a panel of monoclonal antibodies specific for human TCR Vβ families, as detailed in Material and Methods. T cell blasts from each clone were divided in aliquots and stained with each of the monolonaly antibody and the appropriate controls. All clones obtained from two representative patients, named "A" and "B", are represented in the figure.

Figure S2. IFN-γ and IL-17 intra-cellular cytokine staining of plaque-infiltrating T cells of SLE-APS patients. Cells were stained for surface and intracellular markers with the following mAbs for flow cytometry: anti-CD4-PerCP, anti-IL-17-PE, and anti-IFN-γ-FITC (Becton Dickinson). Samples obtained from the atherosclerotic lesions of each of the SLE-APS patients were stimulated with PMA (25 ng/ml) plus 1 μg/ml ionomycin in the presence of brefeldin A (1 μg/ml). The analysis was performed using FACS Canto II (BD), by the acquisition software FACSDiva 6.1.3. For each sample 5000 events were acquired. Dot plots expression of IFN-γ^+ and IL-17^+ on $CD4^+$ T cells of the 10 T-cell lines obtained from the 10 SLE-APS patients (named A, B, C, D, E, F, G, H, I, L) are shown.

Figure S3. β2GPI driven IFN-γ and IL-17 secretion by β2GPI-specific atherosclerotic plaque derived T-cells from SLE-APS patients. Atherosclerotic plaque-derived T-cell lines were expanded from SLE-APS patients by addition of IL-2. At day 7, T-cell blasts from each line were stimulated for 48 h with β2GPI or medium, in the presence of irradiated autologous APCs in ELISPOT microplates coated with anti-IFN-γ, anti-IL-17, or anti-IL-4 antibodies. After specific stimulation with β2GPI, a significant proportion of SLE-APS atherosclerotic plaque-derived Th cells produced IL-17 and IFN-γ, but not IL-4. Values are the mean ± SD number of SFCs per 10^5 cells over background levels.

Patients	Age	Sex	Treatment	SLEDAI	Autoantibodies	Total clones	Total No. of $CD8^+$ clones	No. of $CD8^+$ clones reactive to β2GPI	Total No. of $CD4^+$ clones	No. of $CD4^+$ clones reactive to β2GPI
A	42	F	a+b	7	c+d+e	38	5	0	33	8
B	56	F	a+b	8	c+d+e+f	34	4	0	30	6
C	51	F	b	7	c+d	33	6	0	27	6
D	50	F	b	5	e	31	1	0	30	8
E	50	F	b	8	c+d+e	32	4	0	28	7
F	54	F	a+b	6	c+d+g	34	3	0	31	7
G	55	F	b	7	c+d-h	35	3	0	32	8
H	48	F	a+b	7	c+d+e	30	3	0	27	7
I	49	F	b	8	c+d+e+h	36	4	0	32	8
L	55	F	a+b	6	c+d	31	4	0	27	6

Table S1. Clinical and lab information of the 10 SLE-APS patients. All the 10 patients shown in this table were affected by SLE-APS and were triple positive for aPL, with high titers serum anti-β2GPI, anti-cardiolipin antibodies and with positivity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.

Patients	Age	Sex	Treatment	SLEDAI	Autoantibodies	Total clones	Total No. of $CD8^+$ clones	No. of $CD8^+$ clones reactive to β2GPI	Total No. of $CD4^+$ clones	No. of $CD4^+$ clones reactive to β2GPI
M	43	F	none	0	none	39	5	0	36	0
N	55	F	none	0	none	37	4	0	33	0
O	51	F	none	0	none	29	7	0	22	0
P	50	F	none	0	none	28	4	0	24	0
Q	50	F	none	0	none	36	4	0	32	0
R	54	F	none	0	none	31	3	0	28	0
S	55	F	none	0	none	38	5	0	33	0
T	48	F	none	0	none	26	3	0	23	0
U	49	F	none	0	none	34	5	0	27	0
V	55	F	none	0	none	32	2	0	30	0

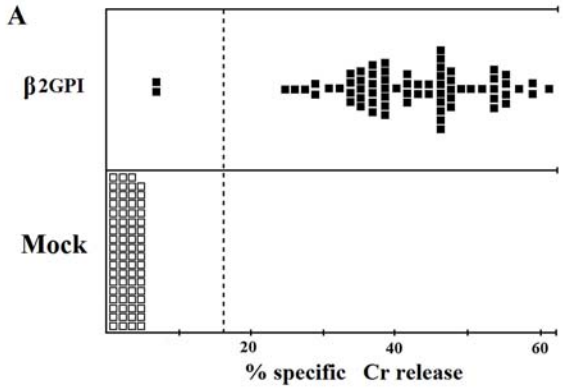
Table S2. Clinical and lab information of the 10 aPL-neg patients. All the 10 patients shown in this table were affected by carotid atherosclerotic arteriopathy but not by SLE-APS and were triple negative for serum aPL, such as anti-β2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant, and they were all negative for any autoantibody.They were not treated with any steroids, nor other immune-suppressants.

Patients	Age	Sex	Treatment	SLEDAI	Autoantibodies	Total clones	Total No. of $CD8^+$ clones	No. of $CD8^+$ clones reactive to β2GPI	Total No. of $CD4^+$ clones	No. of $CD4^+$ clones reactive to β2GPI
Y	44	F	a+b	6	c+d	32	4	0	28	4
YA	53	F	b	9	c+e	28	3	0	24	3
YB	52	F	b	8	c+d+e	25	5	0	20	5
YC	48	F	a+b	7	c+d	34	3	0	31	6
YD	49	F	a+b	5	c+d+e+f	38	6	0	32	7

Table S3. Clinical and lab information of the 5 SLE aPL-pos patients. All the 5 patients shown in this table were affected by SLE but not by APS, although they were positive for aPL, with serum anti-β2GPI, anti-cardiolipin antibodies or with positivity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.

Patients	Age	Sex	Treatment	SLEDAI	Autoantibodies	Total clones	Total No. of $CD8^+$ clones	No. of $CD8^+$ clones reactive to β2GPI	Total No. of $CD4^+$ clones	No. of $CD4^+$ clones reactive to β2GPI
Z	52	F	b	7	c+d+e	29	5	0	26	0
ZA	49	F	a+b	9	c+e+g	34	4	0	28	0
ZB	51	F	a+b	8	c+e+h	31	7	0	24	0
ZC	44	F	b	6	c+h+g	39	6	0	33	0
ZD	56	F	b	7	c+e+h	33	8	0	25	0

Table S4. Clinical and lab information of the 5 SLE aPL-neg patients. All the 5 patients shown in this table were affected by SLE but not by APS, and they were triple negative for serum aPL, such as anti-β2GPI, anti-cardiolipin antibodies and with negativity for Lupus Anticoagulant. a: treated with hydroxychloroquine; b: treated with glucocorticoids; c: ANA-positive; d: anti-SSA-positive; e: anti-ds DNA-positive; f: anti-SSB-positive; g: anti-U1 RNP-positive; h: anti-Sm-positive.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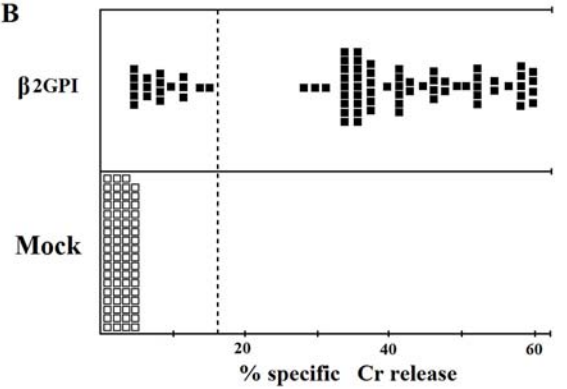
β2GPI
Mock
20
40
60
% specific Cr release


B
β2GPI
Mock
20
40
60
% specific Cr release