Immunological features of primary anti-phospholipid syndrome in connection with endothelial dysfunction

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Objectives. To describe how certain peripheral immune parameters reflect the inflammatory alterations in patients with primary APS.

Methods. Twenty-eight patients with newly diagnosed primary APS were studied. The control group included 26 patients with stable coronary disease and 38 healthy individuals. Peripheral blood lymphocyte subgroups were quantified, intracellular cytokines were measured by flow cytometry, soluble cytokines and auto-antibodies were assessed using ELISA. Endothelial dysfunction was evaluated by measuring endothelium-dependent (flow-mediated; FMD) vasodilation. Carotid duplex ultrasound was performed to quantify the carotid artery intima-media thickness (IMT). Stiffness parameters, augmentation index (Alx) and pulse wave velocity (PWV) were assessed by TensioClinic technology.

Results. Serum IL-4 and IL-6 levels were significantly higher in APS. CD4+IL10+ and CD8+IL10+ cell percentages in APS were significantly increased compared with controls. Th0 and T cytotoxic 0 cell percentages were significantly decreased in patients compared with controls. FMD in APS was significantly lower, while IMT was higher than that of controls. FMD showed strong association with stiffness parameters, Alx and PWV. A significant negative linear correlation was detected between PWV and CD8+IL10+ cell percentages and significant positive linear correlation was found between PWV and CD8+IL10- cell percentage.

Conclusion. In APS, the orchestrated pro-inflammatory cascade can eventually result in endothelial dysfunction, leading to the characteristic vascular abnormalities of the disease.

Key Words: Primary anti-phospholipid syndrome, Peripheral lymphocytes, Circulating and intracytoplasmic cytokines, Th1/Th2 balance, Endothelial dysfunction.

Introduction

APS is an autoimmune prothrombotic disorder characterized by the presence of arterial or venous thromboses and/or recurrent fetal loss and associated with auto-antibodies to epitopes present on phospholipid-binding proteins [1]. Humoral autoimmune processes, such as β2glycoprotein I (β2GPI)-dependent aPL antibodies have important role in the development of thrombosis and further clinical features of APS [2]. β2GPI is a plasma glycoprotein that binds various negatively charged substances, including phospholipids, lipoproteins, activated platelets and activated endothelial cells [3, 4]. Pathological endothelial activation seems to perpetuate the prothrombotic state of APS [5, 6]; additionally, anti-β2GPI antibodies bind to endothelial surfaces through adhered β2GPI, leading to a pro-coagulant and pro-inflammatory phenotype [5, 6]. Many hypotheses have been proposed on various mechanisms in the development of thrombophilia caused by aPL antibodies [7–9]. The pathogenic role of aPL antibodies in APS has been demonstrated in several experimental models [10–12]. Moreover, β2GPI was described to reside in human atherosclerotic plaques along with CD4+ lymphocytes [13]. Although antigenic specificity and functional characteristics of aPL antibodies have been extensively analysed, little is known about the cellular mechanisms that induce the production of aPL antibodies. Anti-β2GPI antibodies in APS patients have been shown to be mainly of IgG and IgA isotype [14]. Probably their reside in human atherosclerotic plaques along with CD4.

Another important cytokine, IL-6 is capable of inducing the final maturation of B-cells into immunoglobulin-secreting plasma cells upon previous activation by IL-4. By plasma cell activation it significantly stimulates the secretion of antibodies, and therefore may contribute to the humoral pro-inflammatory processes in APS [24, 25].

On the other hand, serum IL-10 levels were decreased in patients with APS. IL-10 during the initial activation delivers negative signals that promote the apoptosis of B cells [26], and therefore the reduced serum level of the cytokine may down-modulate this important counter-regulatory, immune-suppressive process. Moreover, IL-10 inhibits the production of several cytokines, such as IL-4, IL-5 and IFN-γ, and also reduces the secretion of growth factors and chemokines, and therefore acts as a key counter-regulator of autoimmune processes. Decreased IL-10 levels can therefore be associated with the impaired negative regulation of pro-inflammatory cytokines and deficiency mice result in increased titres of circulating aPL antibodies and the development of APS manifestations [15].

Besides the pathogenic role of aPL antibodies, pro-inflammatory cytokines and also chemokines have been described to play a significant role in the pathogenesis of APS [16]. A remarkable experiment further underlined the importance of cytokines, namely the injection of soluble receptors with blocking TNF-α activity had a protective effect against the induction of abortions by aPL [17]. Amongst chemokines, the chemokine scavenger receptor D6 has also been implied in the pathogenesis of the disease [18]. Meroni [19] has described that the immune-inflammatory background of APS includes mediators of the innate immunity, also adaptive immune responses specific for self-PL-binding proteins.

IL-4 (B-cell stimulation/differentiation/growth factor) stimulates both activated B- and T-cell proliferation and the differentiation of CD4+ T-cells into Th2 cells. IL-4 induces B-cell activation and class switching [20], and promotes the proliferation and differentiation of activated B-lymphocytes [21, 22]. IL-4 plays a pivotal role in the perpetuation of endothelial dysfunction, exaggerated atherosclerosis, and subsequently the development of arterial and venous thromboses.

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lymphocyte activation, which leads to the perpetuation of autoimmune processes in APS [27].

Since in previous studies we have evaluated the angiographical parameters and endothelial dysfunction in patients with APS, we subsequently assessed whether the endothelial dysfunction showed association with peripheral immunological parameters. The aim of the present study was to describe a broad spectrum of T- and B-cell cytokines, to identify lymphocyte subpopulations and activated T cells and to evaluate growth factors and chemokines in patients with APS. We measured the circulating levels of IL-1, IL-4, IL-6, IL-10 and IFN-γ in patients with primary APS. Furthermore, various parameters indicating endothelial dysfunction were assessed in patients with primary APS, such as endothelium-dependent (flow-mediated; FMD) and endothelium-independent (nitrate-mediated; NMD) vasodilation, FMD, measurement of carotid artery intima–media thickness (IMT). Finally, stiffness parameters were evaluated namely augmentation index (AIX) and pulse wave velocity (PWV).

These results were correlated with immune-competent cell and cytokine levels in order to evaluate the immune-inflammatory events leading to endothelial dysfunction in these patients.

Materials and methods

Patients—study population

Twenty-eight patients (12 males and 16 females), newly diagnosed with primary APS, were involved in the present study. The diagnosis was established according to the Miyakis criteria [28]. Venous or arterial vascular events occurred in 12 and 16 patients, respectively, and also one patient had recurrent abortions. Subjects with venous thromboses (12 patients) received acenocumarol therapy alone (four patients), aspirin alone (six patients) or the combination of acenocumarol and aspirin (two patients). Arterial thrombotic events included acute coronary syndrome (two patients), stroke (three patients), peripheral arterial occlusive diseases (seven patients) and valvulopathy (two patients). All these patients were taking statin, ACE inhibitor and aspirin; furthermore, two of them were taking acenocumarol and one also clopidogrel. No vascular events occurred within the last 3 months. The control group included 26 patients with stable coronary disease and 38 healthy individuals. A cohort of age- and sex-matched healthy individuals was used as controls for the angiographical measurements (n = 38). The study population was assessed for major cardiovascular risk factors, including family history of coronary artery diseases, smoking and diabetes mellitus. Blood glucose, cholesterol and triglyceride levels were evaluated in each case. The study was approved by the ethics committee of the University of Debrecen and informed patient consent was obtained before enrolment.

Serum and blood samples were taken prior to introduction of any medical therapy in all cases. Serum samples were stored at −70 °C until further processing, while cell samples were processed immediately.

Identification of lymphocyte subpopulations and activated T cells

In order to determine lymphocyte subpopulations (T, Th, T cytotoxic, B, NK and NKT cells) and activated T cells isolated from heparinized blood samples, monoclonal antibodies against cell surface markers CD3, CD4, CD8, CD19 and CD56 (BD Biosciences, San Diego, CA, USA and Immunotech, Marseille, France) were used. The expression of T-lymphocyte activation markers such as HLA-DR and CD69 were also determined on CD3+ cells (BD Biosciences). Samples were processed according to the Coulter Q-PREP protocol and system. Measurements were performed and events were collected on Coulter EPICS XL flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

Lyphocytes, monocytes and granulocytes were separated based upon their size and granulation pattern. Lymphocyte subpopulations were quantified as their percentage in the entire population.

Detection of intracytoplasmic cytokines

CD4+ and CD8+ cells isolated from heparinized whole blood were used for intracytoplasmic cytokine measurements, as previously described [29, 30]. Since the cytokine content of resting lymphocytes is very difficult to detect, prior to the labelling process, cells were stimulated using 25 ng/ml phorbol myristate acetate (Sigma Aldrich Corp., St Louis, MO, USA) and 1 ng/ml ionomycin (Sigma Aldrich Corp.) for 4 h at 37 °C and 5% CO2. Excretion of de novo synthesized cytokines from the Golgi apparatus were inhibited by 10 μg/ml brefeldin-A (Sigma Aldrich Corp.). Subsequently, cell surface labelling of CD4 and CD8 antigens was performed using quantum-red conjugated specific monoclonal anti-CD4 and anti-CD8 antibodies (Sigma Aldrich Corp.) at room temperature for 30 min. Red blood cells were eliminated by using FACS Lysis Solution (BD Biosciences), then cell membrane of leucocytes were permeabilized with Permeabilizing Solution (BD Biosciences), for 10 min each, at room temperature in dark. This was followed by washing and labelling of intracytoplasmic cytokines with specific monoclonal antibodies: FITC-labelled anti-human-IFN-γ, phycoerythrin-conjugated anti-human-IL-4 (BD Biosciences), PE-conjugated anti-human-IL-10 (Caltag Laboratories, Burlingame, CA, USA) for 30 min at room temperature in dark. The cells were then fixed with 1% paraformaldehyde solution as the last step of the labelling protocol. Samples were immediately evaluated by flow cytometry. Lymphocytes, granulocytes and monocytes were gated and separated based on their morphological properties.

Determination of serum soluble cytokines

Serum IL-4, IL-10 and IFN-γ was measured by corresponding BD OptEIA ELISA kits (BD Biosciences) according to the manufacturer’s instructions.

Endothelium-dependent (FMD) and endothelium-independent (NMD) vasodilation

Endothelium-dependent vasodilation was assessed with a 7.5-MHz linear array transducer (Sonos 5500; Hewlett-Packard, Soma Technology Inc. CT, USA) by scanning the brachial artery in longitudinal sections, as published previously [31]. The evaluation of endothelial function was always carried out by the same investigator (H.D.) and the data assessment was performed offline by using a digital software (AVITA, Greth Information Systems, IL, USA). All subjects were refrained from smoking and eating for 8 h and were without medications for ≥12 h before the exercise and endothelial function was tested. To minimize mental stress, procedures were performed in a quiet, air-conditioned room (22–25 °C). The left arm was stabilized with a cushion and a sphygmomanometric cuff was placed on the forearm. A baseline image was taken and the blood flow was estimated by time-averaging the pulsed Doppler velocity signals from a mid-artery sample volume. Subsequently, the cuff was inflated to at least 50 mm Hg above systolic blood pressure for 5 min and released rapidly. Post-occlusion diameters were obtained 60 s after deflation. FMD was calculated as the percent change in diameter compared with pre-occlusion values. A mid-artery pulsed Doppler signal was obtained immediately upon cuff release and no later than 15 s after cuff deflation in order to assess hyperemic velocity.

After at least 10 min, another image was taken to reflect the re-established baseline conditions. Diameter measurements were taken at least 3 times at 3- to 4-min intervals after 0.4 mg sublingual nitroglycerin was administered. The maximal FMD and NMD diameters were determined based on the average of
three consecutive diameter measurements as the percent change in the diameter compared with the baseline. Blood flow was calculated by multiplying the velocity time integral with the heart rate and the vessel cross-sectional area. The reproducibility of the FMD assay was excellent. We assessed inter- and intra-assay variability of the assay on a healthy subject. The variability did not exceed 4.6% in both cases, so the accuracy of the method was appropriate according to international recommendations [31, 32].

Carotid duplex ultrasound investigations; measurement of carotid artery IMT

Ultrasound investigations were performed immediately after blood sampling with a colour-coded HP SONOS 5500 carotid duplex equipment (Hewlett Packard Soma Technology Inc. CT, USA, USA) using a 7.5-MHz linear transducer. The analysis included longitudinal and transverse assessment of the carotid arteries. The measurement of the IMT was performed at 10 mm proximal to the carotid bulb or 20 mm proximal to the flow divider. IMT was measured as the distance between the leading edge of the first echogenic line (lumen–intima interface) and the second echogenic line (upper layer of the adventitia) in the far (deeper) artery wall. All measurements were performed at the end of the heart cycle, with the transducer towards the mediolateral direction [25]. Offline analysis was performed using digital video images (AVITA, Gtech Information Systems, IL, USA). Measurements were performed on both common carotid arteries and the larger of the two values was used for data analysis [33].

Assessment of Alx and PWV—stiffness parameters

Measurements were carried out by using a TensioClinic device (Tensionmed Corp., Budapest, Hungary) [34–37]. The measurement is based on the fact that the contraction of the heart causes pulse waves in the aorta. The first wave reflects on the aorta at the bifurcation; therefore, during systole the second wave is easily detectable and a late systolic peak appears. The second reflected wave depends on the stiffness of the large artery, the time (RT S35) spent and the peripheral resistance-dependent amplitude. Alx can be calculated by the amplitude of the reflected and the first wave, which is the pressure difference between the late-systolic peak pressure and the early-systolic peak pressure divided by the late systolic peak pressure. The TensioClinic arteriograph can assess this parameter from the oscillometric data obtained from the 35 mmHg suprasystolic pressure of the brachial artery [34–37].

PWV

PWV is the quotient between the jugular fossa and symphysis distance and the reflection time (RT S35) is the reflection time at 35 mmHg suprasystolic pressure. A jugular fossa–symphysis distance is anatomically identical with the distance between the aortic trunk and the bifurcation. In order to have reproducible results, the patients had to rest at least for 5 min before the measurement, and also the investigation room was completely quiet without any disturbing effects [34–37].

Statistical analyses

Statistica for Windows, version 7.0 (StatSoft), was used for data analysis. Values are presented as mean and s.d. To increase sensitivity of the flow cytometry and to analyse the distribution of the data we used Kolgomorov–Smirnov test. In cases of normal distribution, we determined mean ± s.d. values and used two-sample t-test for statistical evaluation of the experimental data. When the distributions were different from that of normal, median, minimum and maximum values were calculated, and Mann–Whitney test was used. Differences were considered statistically significant at $P < 0.05$. To analyse association between different parameters, Spearman or Pearson correlation tests were used.

Results

Angiological parameters

Patients with APS vs healthy controls. Regarding cardiovascular risk factors (age, systolic- and diastolic blood pressure, plasma level of cholesterol and triglyceride, smoking habits, BMI), there was no significant difference between the two groups. FMD in patients with primary APS was significantly lower than that of controls (3.57 ± 3.3% vs 8.38 ± 4.03%; $P < 0.0001$). However, no significant difference was found in NMD values between the two groups (14.94 ± 7.33% vs 17.49 ± 6.95%). Carotid artery IMT was significantly higher in primary APS patients than in controls (0.681 ± 0.16 mm vs 0.58 ± 0.08 mm; $P = 0.0066$). We found plaques on the carotid artery of 15 patients, yet none caused significant stenosis. Similarly to our early data we found strong negative linear correlation between FMD and carotid artery IMT ($R = -0.718$, $P = 0.0001$). Interestingly, FMD showed strong association with stiffness parameters, Alx and PWV. Negative linear correlation was detectable between these parameters (FMD–Alx: $R = -0.594$, $P < 0.001$; FMD–PWV: $R = -0.655$, $P = 0.0002$). Carotid artery IMT correlated also with stiffness parameters (IMT–Alx: $R = 0.589$, $P = 0.0012$; IMT–PWV: $R = 0.6$, $P = 0.0008$) (Fig. 1).

In order to characterize the immune status of our patients, we assessed the following parameters: number and percentage of T, Th (T-helper), Tc (cytotoxic T cell), NKT (natural killer T-cell), B cells and NK (natural killer) cells, on CD3+ cells, markers of activation (HLA-DR and CD69), intracellular IFN-γ, IL-4 and IL-10 expression of in vitro stimulated CD4+ and CD8+ cells, and soluble IL-1, IL-4, IL-6, IL-8, IL-10 and IFN-γ cytokine levels.

Tcells, T-cell subsets, Bcells

Patients with APS vs controls. We found significant differences in peripheral blood double positive CD4+IL10+ (17.63 ± 12.97% vs 4.32 ± 5.83%) and double positive CD4+IL10+ (17.9 ± 10.76% vs 6.44 ± 7.24%) cell percentages in APS patients when compared with the control group (Fig. 2). The phenotyping of the basic cellular composition of APS patients and patients with stable coronary disease failed to show significant differences in peripheral CD3+, CD4+, CD8+, CD9+, CD56+ and CD3+CD56+ cell percentages (70.3 ± 10.23% vs 69.5 ± 10.69%, $P = 0.91$; 44.5 ± 6.16% vs 44.27 ± 11.28%, $P = 0.95$; 23.2 ± 10.28% vs 22.42 ± 11.03%, $P = 0.84$; 10.5 ± 5.91% vs 13.5 ± 14.79%, $P = 0.54$ and 19.55 ± 4.3% vs 15.5 ± 7.49%, $P = 0.13$, respectively).

Amongst activated T cells, unlike peripheral CD3+/HLADR+ cells, CD3+/CD69+ cells were represented in increased percentages in APS patients when compared with those found in controls (10.4 ± 7.73% vs 12.23 ± 10.93%, $P = 0.63$ and 1.66 ± 1.61% vs 1.16 ± 1.01%, $P = 0.27$, respectively).

APS patients with venous vs arterial manifestations. Comparing the cell percentages of CD4+IL10+ and CD8+IL10+ T cells in the peripheral blood of APS patients with venous vs arterial manifestations we did not find significant differences between the two subgroups, yet double positive CD4+ (14.61 ± 13.42 and 19.31 ± 12.39 vs 4.32 ± 5.83, $P = 0.0014$ and $P < 0.0001$, respectively) and CD8+ (17.82 ± 12.42 and 17.82 ± 10.56 vs 6.44 ± 7.24, $P = 0.0014$ and $P < 0.001$, respectively) cell percentages were significantly higher in patients with venous and arterial manifestation compared with patients with stable coronary disease (Fig. 2).

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Assessment of Th0/Th1/Th2 cells by intracellular cytokine profiling

Patients with APS vs controls. When analysing the fine functional structure of the immune system of patients with APS, we found that Th 0 (CD4+/IFNγ+/IL4+) and T cytotoxic 0 (CD8+/IFNγ+/IL4+) cell percentages were significantly decreased in patients with APS compared with patients with stable coronary disease (0.54 ± 0.85% vs 1.4 ± 1.68%, \(P=0.008\) and 0.49 ± 0.47% vs 1.46 ± 2.08%, \(P=0.01\)). At the same time,
Th1 and Th2 cell percentages did not differ significantly between these groups (22.78 ± 11.65% vs 24.98 ± 9.48% and 0.31 ± 0.35% vs 0.45 ± 2.08%).

**APS patients with venous vs arterial manifestations.** The percentage of Th0, Tc0, Th1, Th2 cells in APS patients with venous vs arterial manifestations was found comparable between the two subgroups.

**APS patients with venous or arterial manifestation vs patients with stable coronary disease.** The comparison of Th0, Tc0, Th1, Th2 cell percentages in APS patients with venous or arterial manifestations compared with patients with stable coronary artery disease (A). Serum IL-1 and IL-4 levels were significantly increased in APS patients with arterial manifestations compared with patients with stable coronary artery diseases (B).

**Circulating cytokines**

**Patients with APS vs controls.** Of serum-soluble cytokines, IFN-γ was present in higher concentrations in the sera of APS patients compared with patients with stable coronary artery disease (99.89 ± 243.98 pg/ml vs 31.03 ± 69.05 pg/ml). Serum IL-1 levels also presented in higher concentrations in APS patients (9.18 ± 20.2 pg/ml vs 4.89 ± 10.93 pg/ml). Interestingly, IL-4 and IL-6 were found to be present in significantly higher titres in APS patients (31.46 ± 60.69 pg/ml vs 1.4 ± 3.23 pg/ml, P = 0.015 and 24.76 ± 13.94 pg/ml vs 10.23 ± 11.97 pg/ml, P < 0.05). Serum IL-10 and IL-8 levels were decreased in patients with APS compared with stable coronary patients (8.18 ± 10.21 pg/ml vs 27.03 ± 70.18 pg/ml and 79.28 ± 37.7 pg/ml vs 128.65 ± 241.5 pg/ml) (Fig. 3).

**APS patients with venous vs arterial manifestations compared with patients with stable coronary artery disease.** The concentration of circulating cytokines in APS patients with venous or arterial manifestations did not differ significantly. Interestingly, serum IL-1 and IL-4 levels were significantly increased in APS patients with arterial manifestations compared with patients with stable coronary artery diseases (18.36 ± 27.73 pg/ml vs 4.89 ± 10.93 pg/ml, P = 0.05 and 48.71 ± 80.05 pg/ml vs 1.4 ± 3.23 pg/ml, P = 0.0031) (Fig. 3).

**Association between angiological parameters and the immune status**

The evaluation of the relationship between the angiological parameters and immune status of patients with primary APS showed that carotid artery IMT, AIx and PWV had strong positive linear correlation with serum levels of IL-4 (R = 0.7 and P = 0.015; R = 0.7 and P = 0.015; R = 0.899 and P = 0.00016, respectively). An interesting connection was detectable between PWV and CD8+IL10+ and CD8+IL10− cell percentages. We found significant negative linear correlation between PWV and CD8+IL10+ cell percentages (R = −0.395 and P = 0.045) and significant positive linear correlation between PWV and CD8+IL10− cell percentage (R = 0.46 and P = 0.015) (Fig. 4).

**Discussion**

The aim of the present study was to describe how certain peripheral immune parameters reflect the inflammatory alterations and endothelial dysfunction in patients suffering from primary APS.

In patients with APS, we found evidence for endothelial dysfunction compared with healthy individuals, signified by reduced FMD, increased carotid artery IMT [38], also a positive association between AIx and PWV. Presumably, in APS the ongoing arterial and venous endothelial dysfunction leads to exaggerated atherosclerosis, subsequently to the development of arterial and venous thromboses.

When we categorized APS patients to arterial and venous subgroups, we found significantly higher IL-1 levels in patients with arterial manifestations. This could be explained that IL-1-mediated pro-inflammatory processes are characteristic to atherosclerotic manifestations, while these processes do not present in APS patients with pathological venous symptoms. Therefore, although IL-1 levels in the overall APS population is non-significantly increased, when we take out the non-IL-1-driven APS patients with venous manifestations, IL-1 levels in APS patients with arterial manifestations alone reached a significant increase compared with controls. These findings reinforce the fact that IL-1 is presumably a pivotal cytokine in the pathogenesis of APS with arterial, but not with venous manifestations.

We found significantly higher titres of IL-4 and IL-6 in APS patients. Interestingly, markers of endothelial dysfunction, IMT, AIx and PWV were positively correlating with IL-4 levels, suggesting that by the activation of the humoral and cellular immune responses, IL-4 plays a pivotal role in the perpetuation of endothelial dysfunction, exaggerated atherosclerosis, and subsequently the development of arterial and venous thromboses.

Since IL-6 is a strong promoter of humoral autoimmune processes following IL-4 activation, its elevated levels contributes to an exaggerated and disproportional pathogenic aPL autoantibody production [24, 25].

On the other hand, serum IL-10 levels were decreased in patients with APS. As explained previously, decreased IL-10 levels can therefore be associated with the impaired negative regulation of pro-inflammatory cytokines and lymphocyte activation, which
leads to the perpetuation of autoimmune processes in APS [27]. Interestingly, CD4+IL10+ and CD8+IL10+ cell percentages in APS patients were significantly increased, which might be a counter-regulatory effect of the pro-inflammatory cascade in the disease.

Subsequently, endothelial dysfunction was assessed and we found that FMD in APS was significantly lower, while IMT was increased. Moreover, a negative linear correlation was found between FMD and IMT. The fact that FMD showed strong association with stiffness parameters, AIx and PWV further confirmed the ongoing severe endothelial dysfunction and blood vessel stiffness in APS, which signifies the arterial and venous complications of the disease.

Finally, as we assessed the association between peripheral immunological parameters and endothelial dysfunction, we found that IMT, AIx and PWV showed strong positive linear correlation with serum levels of IL-4. Since this cytokine is a key regulator of both humoral and cellular immune responses, this correlation suggests the important role of immune-mediated processes in the development of endothelial dysfunction and blood vessel stiffness.

Interestingly, IL-10-producing CD8+ cells correlated negatively, while CD8+IL10− cells correlated positively with PWV, indicating that this cytokine also significantly contribute to endothelial dysfunction in APS.

Changes in PWV are characteristic to chronic vascular processes and in these measurements signify a constantly ongoing inflammatory damage. During these chronic, pro-inflammatory processes, counter-regulatory machineries try to compensate, in our case characterized by an increase of IL10 production by CD8+ cells. Therefore, the elevated levels of CD8+ intracytoplasmic IL10 can be explained as a response to chronic intravascular inflammation. Since PWV is increased in parallel with vascular damage, as the artery is becoming more rigid and stiff, our finding that CD8+ IL10+ cell percentages show a negative correlation with PWV enables us to assume that a defective counter-regulatory process leads to the development of vascular damage. With regard to the fact that arterial stiffness increases as a result of a pathological vascular remodelling, we believe that this process evolves slower in patients with a more pronounced CD8+ IL10+ counter-regulation.

In patients with APS, three processes are present in parallel. First, an auto-antibody-mediated humoral autoimmune response signified by the presence of various aPL auto-antibodies. Second, a wider, general, more robust atherosclerotic, arterial pro-inflammatory cascade significantly perpetuates the vascular damage. Finally, opposed to the first two pro-inflammatory processes, an induced counter-regulatory machinery tries to balance the disproportional harmful effects. In those patients with APS, where this CD8+IL10+ counter-regulatory effect is prominent, the arterial stiffness therefore becomes milder. Besides the intracytoplasmic CD8+ IL10+ counter-regulation, the fact that we found reduced serum IL10 levels compared with controls, further reinforces the fact that in APS the IL10-mediated counter-regulatory processes are defective, therefore leading to the perpetuation of vascular damage, signified by increased arterial stiffness and therefore elevated PWV values.
We pointed out that immunological processes take place in different pathways, in a more complex fashion in those clinical manifestations of atherothrombosis, where direct B-cell activation presents. Our results imply that besides the generally accepted Th1 pathway, Th2 cytokines play a pivotal role, namely IL-4 and IL-10. Concerning IL-10, we believe that by comparing the IL10+/IL10− groups we could show its regulatory features in the angiobiological parameters in APS.

Taking these findings together, we conclude that various immune-competent cells orchestrate the pro-inflammatory cascade, which eventually leads to disproportional cytokine balance and an activated circulating lymphocyte-cell pool in APS. This chronic pro-inflammatory process subsequently leads to endothelial dysfunction and finally to the development of arterial and venous thromboses. By pinpointing the major players of these cascades, we can target the propagation of this pathogenic inflammation; novel biological targets can be identified (e.g. IL-4, IL-6, IL-10 cytokine targeting) and innovative biological therapies can be designed that eventually could lead to the deceleration of immune-mediated endothelial dysfunction in patients with APS. We believe that besides conventional anti-platelet and anti-thrombotic therapy in APS, unique biologics will get in to the future therapeutic regime of the disease.

**Rheumatology key messages**

- APS is an autoimmune disorder characterized by the presence of thromboses and endothelial dysfunction.
- In APS, the assessment of immune parameters suggested an orchestrated pro-inflammatory cascade.
- This eventually results in endothelial dysfunction, leading to the characteristic vascular abnormalities.

**Disclosure statement:** The authors have declared no conflicts of interest.

**References**


