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*J Immunol* published online 17 May 2010
http://www.jimmunol.org/content/early/2010/05/17/jimmunol.0902765

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Effects of Polyclonal IgG Derived from Patients with Different Clinical Types of the Antiphospholipid Syndrome on Monocyte Signaling Pathways

Anastasia Lambrianides,*† Christopher J. Carroll,* Silvia S. Pierangeli,‡ Charis Pericleous,*‡ Ware Branch,§ Jurhee Rice,§ David S. Latchman,* Paul Townsend,* David A. Isenberg, † Anisur Rahman, † and Ian P. Giles*‡

A major mechanism of hypercoagulability in the antiphospholipid syndrome (APS) is antiphospholipid Ab-mediated upregulation of tissue factor (TF) on monocytes via activation of TLRs, p38 MAPK, and NF-κB pathways. We examined whether monocyte signaling pathways are differentially activated by IgG from patients with vascular thrombosis (VT) alone compared with IgG from patients with pregnancy morbidity (PM) alone. We purified IgG from 49 subjects. A human monocyte cell line and ex vivo healthy monocytes were treated with 100 μg/ml IgG for 6 h, and cell extracts were examined by immunoblot using Abs to p38 MAPK and NF-κB. To further investigate intracellular signaling pathways induced by these IgGs, specific inhibitors of p38 MAPK, NF-κB, TLR4, and TLR2 were used to determine their effect on TF activity. Only IgG from patients with VT but no PM (VT+/PM−) caused phosphorylation of NF-κB and upregulation of TF activity in monocytes. These effects were not seen with IgG from patients with PM alone (VT−/PM+), anti-phospholipid Ab-positive patients without APS, or healthy controls. TF upregulation caused by the VT+/PM− samples was reduced by inhibitors of p38 MAPK, NF-κB, and TLR4. The effects of VT+/PM− IgG on signaling and TF upregulation were concentrated in the fraction that bound β2-glycoprotein I. Our findings demonstrate that IgGs from patients with diverse clinical manifestations of APS have differential effects upon phosphorylation of NF-κB and p38 MAPK and TF activity that may be mediated by differential activation of TLR4. The Journal of Immunology, 2010, 184: 000–000.

The antiphospholipid syndrome (APS) is diagnosed in patients who suffer vascular thrombosis (VT) and/or pregnancy morbidity (PM) in association with persistently positive blood tests for anti-phospholipid Abs (aPLs) (1, 2). APS is the most common cause of acquired venous and arterial thrombosis (3) and the most important treatable cause of recurrent miscarriage (4). Prospective clinical studies have shown a significant association between aPLs and arterial and venous thrombosis (5) as well as PM (6). Patients with APS develop a wide range of clinical manifestations (7), and pathogenic aPLs have been shown to exert their thrombotic effects through interactions with endothelial cells (ECs) (8), platelets (9), and monocytes (10).

aPLs are commonly identified by the anticardiolipin (aCL) ELISA, anti–β2–glycoprotein I (β2GPI) ELISA, and lupus anticoagulant (LA) assay (2). Some patients who test positive in these assays will develop VT, others will develop PM, some will have both, and some will develop neither despite the persistent presence of serum aPLs (7). Fewer than 4.2% of patients with PM due to APS go on to develop VT (11, 12). This study investigates the hypothesis that aPLs present in these VT−/PM+ patients lack the ability to act on target cells to promote thrombosis. In particular, we compared the ability of polyclonal IgG from VT+/PM− or VT−/PM+ patients with APS to increase the activity of tissue factor (TF), the major initiator of coagulation produced by monocytes.

TF expression is increased in monocytes from patients with APS (13, 14) and on healthy monocytes exposed to aPLs in vitro (15, 16). This aPL-mediated upregulation of TF in monocytes occurs via ERK-1, p38 MAPK, and NF-κB signaling pathways (10, 17). Similarly, aPL-mediated activation of p38 MAPK and NF-κB pathways has been shown in cultured ECs (18), and TLR4 has been implicated in this process by both in vitro (19) and in vivo (20) studies. aPLs react with the β2GPI–TLR4–Annexin A2 complex in human monocyte plasma membranes (21). TLR2 is implicated in the inflammatory activation of mouse fibroblasts by human aPLs, but there are no previous studies of the effects of aPLs on TLR2 in monocytes (22).

Previous studies (10, 17) of the effects of aPLs upon monocytes have tested samples of purified polyclonal aPLs from limited numbers of patients with mostly VT alone. Only one study, however, has clearly examined large numbers of patients with...
different manifestations of APS: VT alone and PM alone (23). Interestingly, by proteomic analysis of monocytes isolated from 51 patients with APS, this group identified the differential expression of several monocyte proteins between the different clinical subgroups, thus reinforcing our hypothesis that aPLs from patients with different clinical manifestations of APS may have differential effects upon target cells. In this study, we compared the effects of a large number of polyclonal IgG samples, derived from different APS patient subsets and control groups, on TLR, p38 MAPK, and NF-κB signaling pathways as well as TF function in a human monocyte cell line and ex vivo healthy monocytes.

Materials and Methods

Patients

Serum samples from 49 individuals were obtained for this study from patients under our care at University College London Hospital, London, U.K., and through Prof. Silvia Pierangeli at University of Texas Medical Branch, Galveston, TX, and Dr. Warren Branch at University of Utah Health Services Center, Salt Lake City, UT. All of the subjects signed consent forms approved by the local ethics committees at each institution. Of 27 patients fulfilling the classification criteria for APS (2), 10 had a history of VT alone (VT+/PM−), 7 had both VT and PM (VT+/PM+), and 10 had experienced only PM (VT−/PM+). Serum samples from patients were collected after the clinical event and stored at −80°C. Twelve patients (nine with systemic lupus erythematosus [SLE]) were aPL-positive but lacked APS (aPL+/APS−), and 10 healthy individuals were aPL- and APS-negative (aPL-negative).

Purification and immunological characterization of IgG

IgG was purified from all of the serum samples by protein G-Sepharose affinity chromatography (GE Healthcare Life Science Buckinghamshire, U.K.), passed through Detoxi-Gel Endotoxin Removing Columns (Fisher Scientific, Leicestershire, U.K.), and confirmed to be endotoxin-free (<0.06 endotoxin units per milligram by the Limulus amebocyte lysate assay [Bio-Rad, Hercules, CA]). The concentration of purified IgG was determined using the Nanodrop ND-1000 Spectrophotometer (Labtech International, East Sussex, U.K.). The aCL and anti-β2GPI activity of IgG was measured as previously described (24) using international calibrators in G phospholipid units (GPLU), from Louisville APL Diagnostics, Seabrook, TX) and the IgG Sapporo standard, no. 508668, HCAL (Centers for Disease Control and Prevention, Atlanta, GA) (2). LA activity was measured by clinical assays in the routine hospital laboratory using the dilute Russell’s viper venom test time.

To establish the effects of exposing monocytes to IgG, initially we used pooled samples from the four groups of subjects VT+/PM−, VT−/PM−, aPL+/APS−, and aPL-negative. To ensure reproducibility of results, we tested two different pooled samples, obtained by combining an equal concentration of IgG from several individual samples, for each of the four groups. In each group, the first pooled sample was derived from five individuals, and the second was derived from seven individuals (two subjects were common to both pools in each group). aCL and anti-β2GPI binding of each pooled IgG sample were tested at concentrations of 100 μg/ml. Pooled IgG from both VT+/PM− and VT−/PM− groups had high aCL (≥52 GPLU) and anti-β2GPI (>136% binding compared with a concentration of 100 μg/ml HCAL) binding. Pooled IgG from the aPL+/APS− groups had moderate aCL (33 GPLU) but low anti-β2GPI (26% binding compared with a concentration of 100 μg/ml HCAL) activity, whereas pooled IgG from the aPL-negative group did not bind either CL or β2GPI.

Affinity purification of APS IgG that binds β2GPI

Human β2GPI (1.5 mg) (Louisville APL Diagnostics) was coupled to 1 ml cyanogen bromide-activated Sepharose 4B (Pharmacia, Milton Keynes, U.K.). Total IgG fractions from APS patients were applied to the β2GPI column and incubated overnight at 4°C, after which the column was washed. Eluted 1-ml fractions containing IgG anti-β2GPI Abs were obtained by applying 0.1 M glycine (pH 2.7) and immediately neutralized with 100 ml 1 M Tris (pH 9.0). Eluted fractions were concentrated (YM-30 centrifugal filter devices, Millipore, Bedford, MA) and resuspended in PBS (pH 7.4). In our hands, this technique enabled us to obtain 200 μg of purified anti-β2GPI such that the binding of anti-β2GPI in the affinity-purified fraction is more than twice as high as that in the residual sample that did not bind the column.

Isolation of healthy monocytes

Peripheral venous blood samples from a healthy donor were used to isolate mononuclear blood cells by Ficoll-Paque PLUS (GE Healthcare Life Science) density gradient centrifugation. Monocytes were purified using the immunomagnetic EasySep Human CD14 Positive Selection Protocol (StemCell Technology, San Carlos, CA) and cultured with serum-free RPMI 1640 medium and treated with 100 μg/ml purified IgG or 3 μg/ml LPS for 6 h. The 6-h incubation period has been shown to be appropriate for experiments addressing the effects of aPLs on monocytes by López-Pedrera et al. (10, 23) and was also consistent with the results of our time-course experiments on the U937 cell line (see below).

In vitro exposure of monocytes to IgG and inhibitors

The human promonocytic (U937) cell line (European Collection of Cell Cultures, Salisbury, U.K.), derived from a patient with generalized histiocytic lymphoma (25), was maintained in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. U937 cells (1 × 105 cells per milliliter) were incubated with 100 μg/ml purified IgG, 3 μg/ml LPS, or 100 ng/ml TNF-α for time periods between 5 min and 24 h.

In some experiments, cells were pretreated with specific inhibitors for 30 min prior to exposure to IgG. Bay 11-7082 (Alexis Biochemicals, U.K.), a specific inhibitor of NF-κB activity, was used at a concentration of 50 μM SB203580 (Calbiochem, U.S.), a specific p38 MAPK inhibitor, was used at a concentration of 1 μM. All of the inhibitors were dissolved in <1% DMSO in PBS. Anti-human TLR2 Ab (eBioscience, Hatfield, U.K.) and Escherichia coli K12 mubB LPS, a TLR4 agonist (InvivoGen, San Diego, CA) were used at concentrations of 1 μg/ml. To confirm specific effects of these inhibitors, we repeated experiments using 1% DMSO/PBS alone or SB204274 (Calbiochem), a nonfunctional analogue of SB203580 in DMSO.

Western blotting for the analysis of NF-κB and p38 MAPK signaling pathways

Cell extracts were prepared by addition of 100 ml lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 10 mM NaF, 1 mM Na3VO4, and complete mini protease inhibitor mixture tablets [Roche, U.K.]). Samples were resolved via 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, blocked, and incubated overnight at 4°C with a 1:1000 dilution of rabbit monoclonal anti-human phosphorylated Ser276 and total NF-κB p65 (Ser276) and phosphorylated (Thr180/Tyr182) and total p38 MAPK, followed by 1 h of incubation in a 1:2000 dilution of HRP-conjugated goat anti-rabbit IgG. Equivalent protein loading was demonstrated using an anti-human actin polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by chemiluminescence (GE Healthcare Life Science), their intensities were quantified by densitometric analysis (QuantityOne software, Bio-Rad, Hercules, CA), and results were expressed as a ratio of relative expression.

Assay for monocyte TF activity

Monocyte TF activity was determined using a chromogenic assay (Acti- chrome TF, American Diagnostica, Stamford, CT) that measures factor Xa after activation by the TF–factor VII complex. U937 cells were treated for 6 h with IgG (100 μg/ml), and cell lysates then were tested for TF activity.

Statistical analysis

Nonparametric statistical analyses were performed. Mean values are shown, and Mann-Whitney-Wilcoxon scores were carried out to compare two groups of unpaired data using Prism software, version 4.0c (GraphPad, San Diego, CA).

Results

Characteristics of subjects and IgG samples

Table I gives the relevant clinical and laboratory features of the 49 subjects. Forty-six (94%) were women. aCL and anti-β2GPI levels and LA positivity were similar in the three APS groups (VT+/PM−, VT−/PM+, and VT+/PM+) but considerably lower in the aPL+/APS− group as expected because higher titers of IgG aCL and IgG anti-β2GPI are markers of increased risk of developing VT or PM (2). The comparable levels of aPL detected by all three assays in the VT+/PM− and VT−/PM−
groups indicate that differences in the functional effects of IgG from these two groups are not likely to be due to differences in levels of aPL.

**Pooled IgG from VT+/PM− patients but not VT−/PM+ patients promoted phosphorylation of NF-κB and p38 MAPK in U937 cells**

To establish the effects of exposing monocytes to IgG, we initially used pooled IgG samples from the four clinical groups VT+/PM−, VT−/PM+, aPL+/APS−, and aPL-negative. U937 cells were treated with pooled IgG for 0, 5, 10, and 15 min and 1, 6, and 24 h. Maximal differences in phosphorylation of NF-κB and p38 MAPK were observed between the different groups after 6 h of exposure to IgG (as shown in Fig. 1). U937 cells exposed to these IgGs or to TNF-α between 0 min and 1 h had very little phosphorylation of NF-κB and p38 MAPK (too little to see differences between untreated cells and positive control), whereas cells cultured for 24 h under any conditions (i.e., exposure to IgG from all of the groups, TNF-α, or even medium alone) showed equal levels of nonspecific phosphorylation of both NF-κB p65 and p38MAPK. After 6 h of incubation, VT+/PM− IgG caused a ∼4-fold increase in phosphorylation of NF-κB compared with that caused by IgGs from the other three groups (p < 0.05) (as shown in Fig. 1A). Similarly, a ∼6-fold increase in phosphorylation of p38 MAPK was seen with the VT−/PM− sample compared with those of the other groups (p < 0.05) (as shown in Fig. 1B). In contrast, IgG from VT−/PM+ patients had no greater effect on NF-κB and p38 MAPK phosphorylation than IgG from the control (aPL+/APS− and aPL-negative) groups or medium alone.

These experiments with pooled samples enabled us to establish the ideal incubation time for further experiments as 6 h of exposure of monocytes to IgG. This finding was consistent with the previous work of López-Pedrera et al. (10, 23). To investigate the biological relevance of our initial findings using pooled samples and U937 cells, we addressed the following questions: Are similar differences between VT+/PM− and VT−/PM+ samples seen in monocyte signaling when IgG from individual subjects is tested? Are similar effects seen when ex vivo monocytes rather than U937 cells are used? What is the functional consequence of the 6 h of exposure to IgG?

**Similar profiles of NF-κB and p38 MAPK phosphorylation occurred in U937 cells and ex vivo monocytes exposed to IgG from individual subjects**

We examined the effects of IgG from individual patients chosen at random from the four groups previously tested (VT+/PM−, VT−/PM+, aPL+/APS−, and aPL-negative). In general, the same pattern of phosphorylation found with pooled IgG was observed with individual IgG samples. NF-κB p65 (Fig. 2A) and p38 MAPK (Fig. 2B) phosphorylation in U937 cells exposed to IgG from VT+/PM− patients were ∼3-fold higher (p < 0.05) than those from the other groups.

To investigate the possibility that differences in NF-κB and p38 MAPK phosphorylation between groups were due to differences in titer of aPL, we plotted aPL level against each of these outcome measures for all 28 samples in the VT+/PM−, VT−/PM+, VT+/PM+, and aPL+/APS− groups (Fig. 3A, B). There was no correlation between aPL titer and either phosphorylation of NF-κB (r² = 0.0003117) or p38 MAPK (r² = 0.03016).

We then studied the effects of IgGs from four individual patients chosen at random upon ex vivo monocytes isolated from a healthy donor (Fig. 4A). We found a similar profile of IgG-mediated NF-κB p65 and p38 MAPK phosphorylation in ex vivo monocytes compared with what we previously found in U937 cells (Figs. 1, 2). Therefore, IgG from patients with VT+/PM− increased NF-κB p65 and p38 MAPK (Fig. 4B, C) phosphorylation compared with IgG from patients with VT−/PM+, although it only reached statistical significance (p < 0.05) for NF-κB p65.

**TF activity in U937 cells was stimulated by VT+/PM− IgG but not by VT−/PM+ IgG and this stimulation is blocked by inhibiting NF-κB, p38 MAPK, or TLR4 signaling pathways**

Exposure to pooled VT+/PM− IgG caused a ∼4-fold increase in TF activity (p < 0.05) compared with those caused by VT−/PM+, aPL+/APS−, or aPL-negative IgGs (Fig. 5A). In contrast, VT−/PM+ IgG did not significantly increase TF activity compared with aPL+/APS− or aPL-negative IgG.

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**Table I. Clinical and laboratory features of the subjects used as a source of IgG**

<table>
<thead>
<tr>
<th>Feature</th>
<th>VT+/PM− (n = 10)</th>
<th>VT−/PM+ (n = 10)</th>
<th>VT+/PM+ (n = 7)</th>
<th>aPL+/APS− (n = 12)</th>
<th>aPL−Negative (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean y ± SEM)</td>
<td>51.8 ± 4.8</td>
<td>57.1 ± 3.3</td>
<td>45.3 ± 3.4</td>
<td>50.4 ± 4.4</td>
<td>33.6 ± 2.9</td>
</tr>
<tr>
<td>Sex</td>
<td>Male 9/Female</td>
<td>10 Female</td>
<td>7 Female</td>
<td>1 Male/11 Female</td>
<td>1 Male/Female</td>
</tr>
<tr>
<td>PAPS</td>
<td>5 (50%)</td>
<td>7 (70%)</td>
<td>6 (86%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SAPS</td>
<td>5 (50%)</td>
<td>3 (30%)</td>
<td>1 (14%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other ARD</td>
<td>5 SLE</td>
<td>3 SLE</td>
<td>1 SLE</td>
<td>9 SLE</td>
<td></td>
</tr>
<tr>
<td>Live births</td>
<td>8</td>
<td>17</td>
<td>7</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Total APS-related PM</td>
<td>0</td>
<td>20 (10 FT, 10 ST,</td>
<td>13 (4 FT, 6 ST,</td>
<td>13 (4 FT, 6 ST,</td>
<td></td>
</tr>
<tr>
<td>Arterial VT</td>
<td>5 (3 stroke, 5 TIA)</td>
<td>0</td>
<td>6 (5 stroke)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Venous VT</td>
<td>5 (4 DVT, 1 PE)</td>
<td>0</td>
<td>6 (4 DVT, 3 PE)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LA-positive</td>
<td>8 (80%)</td>
<td>9 (90%)</td>
<td>6, INT</td>
<td>3 (25%)</td>
<td>0</td>
</tr>
<tr>
<td>aCL (mean)</td>
<td>62.5 ± 8.8</td>
<td>52.2 ± 9.5</td>
<td>72.2 ± 12.2</td>
<td>33.4 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>GPLU ± SEM</td>
<td>117 (0.90 ± 0.12)</td>
<td>136 (0.83 ± 0.09)</td>
<td>90 (0.69 ± 0.15)</td>
<td>26 (0.18 ± 0.06)</td>
<td>0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Warfarin</td>
<td>10</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Immunosuppressives</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

aAnti-β2GPI activity was calculated as mean percentage binding to a concentration of 100 μg/ml HCAL.

bIn the VT−/PM− group, one patient was taking 7 mg prednisolone, a second was taking 10 mg prednisolone and 100 mg azathioprine. In the VT+/PM+ group, one patient was taking 4mg prednisolone and 50 mg azathioprine, whereas another patient was taking 400 mg hydroxychloroquine. Two of the VT+/PM− were given warfarin on clinicians judgment for primary prevention and/or warfarin-responsive headache with normal brain scan.

ARD, autoimmune rheumatic disease; DVT, deep vein thrombosis; FT, first trimester; LA, lupus anticoagulant; NT, not tested; PAPS, primary antiphospholipid syndrome; PE, pulmonary embolus; PL, pregnancy loss; SAPS, secondary antiphospholipid syndrome; SLE, systemic lupus erythematosus; ST, second trimester; TIA, transient ischemic attack.
Fig. 5B shows the effects of inhibitors of p38 MAPK (SB203580), NF-κB (Bay 11-7082), and TLR4 (E. coli K12 msbB LPS) and of anti-TLR2 Ab on TF activity induced by the IgG samples. The low levels of TF activity in cells exposed to IgGs from the VT+/PM+, aPL+/APS−, and aPL-negative groups were not affected by any of the inhibitors. The increase in TF activity stimulated by VT+/PM− IgG was significantly reduced by inhibitors of p38 MAPK (p = 0.01), NF-κB (p = 0.01), and TLR4 (p = 0.01) but not anti-TLR2 Ab (p = 0.06). Addition of either DMSO alone or a nonfunctional analogue of SB203580 did not have any appreciable effects on TF activity (data not shown).

**Stimulatory effects of VT+/PM− IgG on NF-κB phosphorylation are concentrated in the IgG fraction that binds β2GPI**

Affinity purification of anti-β2GPI Abs from a total IgG pooled sample of four VT+/PM− patients confirmed that the anti-β2GPI subfraction is responsible for increased phosphorylation of NF-κB p65 (Fig. 6). In contrast, the anti-β2GPI−depleted IgG fraction showed an appreciable reduction in ability to promote NF-κB p65 phosphorylation compared with those of both the anti-β2GPI−enriched and whole IgG fractions. Anti-β2GPI activity was calculated as mean percentage binding to a concentration of 100 μg/ml HCAL and was found to be 131 for the anti-β2GPI affinity-purified IgG sample and 65 for the anti-β2GPI−depleted IgG.

What are the effects on monocytes of IgG samples from APS patients who have both VT and PM (VT+/PM+)?

The results of testing IgG from seven VT+/PM+ individuals are included in Fig. 2. Three of these seven VT+/PM+ IgG samples showed the greatest ability to cause p38 MAPK phosphorylation and the greatest NF-κB phosphorylation. These samples resemble VT+/PM− samples. The other four VT+/PM+ samples had far less effect on either signaling pathway and resemble VT−/PM+ samples. There was no correlation with the most recent clinical event. In four cases, the most recent event was PM, in one it was VT, and in two VT and PM had occurred during the same clinical episode. We found a similar spread of results when we tested the effect of seven individual VT+/PM+ samples on TF activity in monocytes. The results for these seven samples were 1, 3.3, 6.8, 11.8, 18.5, 32.5, and 54 (TF activity in pM, results are mean of two separate experiments).

**Discussion**

We have shown that IgG from VT+/PM− patients promotes upregulation of monocyte TF activity that is prevented by specific inhibitors of p38 MAPK and NF-κB. In contrast, IgG from VT−/PM+ patients could not promote phosphorylation of these signaling pathways.
molecules or stimulate upregulation of TF activity. These functional differences were seen despite the fact that the VT+/PM+ and VT+/PM− samples had similarly high levels of CL binding and β2GPI binding.

Previous studies on monocytes (10, 13, 17, 21) tested polyclonal aPL samples from limited numbers of patients with VT alone. Two groups (10, 26) purified total IgG from seven patients with VT+/PM−, whereas another study (21) used polyclonal IgG anti-β2GPI Abs from three patients with VT+/PM−. We investigated IgG from larger numbers of subjects and are the first to compare effects of purified IgGs from VT+/PM− and VT−/PM+ patients with APS. The only previous studies to compare VT+/PM− and VT−/PM+ groups looked at ex vivo monocytes (10, 23) and showed that monocytes extracted from APS patients with VT (including both VT+/PM− and VT+/PM+) had higher TF mRNA, higher surface expression of TF, and altered signaling compared with those of monocytes from VT+/PM+ APS patients, aPL-negative patients with VT, and healthy controls (10). Furthermore, monocytes from patients with APS and thrombosis have higher levels of vascular endothelial growth factor and tyrosine kinase vascular endothelial growth factor receptor 1 than those from patients with APS and no thrombosis or healthy controls (26). Proteomic analysis and mass spectrometry identified six proteins whose expression was significantly different in monocytes from the APS/VT group than those in the three other groups. They showed the same results of TF expression, signaling, and proteomics in monocytes from healthy people exposed to purified IgG from patients with APS, but these IgG samples were derived only from patients with VT and were pooled before addition to the monocytes. Our results agree with and complement those of López-Pedrera et al. (10). We also found increased TF and increased p38 MAPK and NF-κB signaling in monocytes exposed to VT+/PM− IgG but have further demonstrated that purified VT+/PM+ IgG does not cause similar effects. This finding is consistent with the fact that those effects have not been seen on monocytes derived from VT−/PM+ patients (9). López-Pedrera et al. studied TF expression, whereas we studied activity. Expression and activity of TF are not linked directly because cell surface TF may be inactive due to encryption or complex formation with inhibitors. In future studies, we will measure both activity and expression because it is now recognized that TF may have pathogenic proinflammatory effects in APS separate from the effects on thrombosis that are measured in the activity assay (27, 28).

The distinct differences in IgG-mediated phosphorylation of monocyte signaling molecules that we observed at 6 h support the findings of López-Pedrera et al. and other groups who have examined the time course of aPL-mediated signaling activation in platelets and HUVECs (18). We then confirmed the biological significance of this 6 h exposure to IgG by correlating these differences in signaling with our findings from the TF activity assay.

**FIGURE 3.** Effects of IgG on monocyte activation are not related to the aPL titers. aPL titers of all 28 patients in the VT+/PM−, VT−/PM+, VT+/PM+ and aPL+/APS− groups (not healthy controls, who have no aPL) against both (A) p38 MAPK (\( r^2 = 0.03016 \)) and (B) NF-κB p65 (\( r^2 = 0.003117 \)) phosphorylation show no correlation between aPL titer and either outcome.

**FIGURE 4.** IgGs from individual patients display similar patterns of activation of p38 MAPK and NF-κB on ex vivo monocytes. Western blot analysis of cell lysates from healthy monocytes treated with IgG (100 μg/ml) or LPS (3 μg/ml) for 6 h. A. Representative blot with Abs specific for human phosphorylated and total proteins against NF-κB p65 and p38 MAPK. The positive control (LPS) in lane 1 shows stimulation of p38 MAPK and NF-κB p65. Four individual patients from the VT+/PM− group are shown in lanes 2–5, four individual patients from the VT−/PM+ group are shown in lanes 6–9, four individual patients from the aPL+/APS− group are shown in lanes 10–13, and a pooled IgG sample from four healthy controls (aPL-negative) is shown in lane 14. B. Quantitative densitometric analysis of the blots shown in A displaying ratio of phosphorylated to total protein against NF-κB. C. Quantitative densitometric analysis of the blots shown in A displaying ratio of phosphorylated to total p38 MAPK. Statistically significant differences are shown (\( * p < 0.05 \)).
FIGURE 5. APS IgG activates monocyte TF activity. A, Only pooled VT+/PM+ IgG activates monocyte TF activity. U937 cells were treated with 100 \( \mu \)g/ml pooled IgG from four clinical groups (VT+/PM−, VT−/PM+, aPL+/APS−, and aPL-negative) or 3 \( \mu \)g/ml LPS for 6 h. Cells were lysed, and TF activity (pM) was determined using the Actichrome TF assay. Values represent the mean and SE of three independent experiments. Statistically significant differences are shown (\( p < 0.05 \)). B, APS IgG activates monocyte TF activity via the NF-\( \kappa \)B, p38 MAPK, and TLR4 pathways. U937 cells were pretreated for 1 h with 1 \( \mu \)M SB203580, 50 \( \mu \)M Bay 11-7082, 1 \( \mu \)g/ml anti-TLR2 blocking Ab, or 1 \( \mu \)g/ml TLR4 antagonist (E. coli K12 msbB LPS), then treated with 100 \( \mu \)g/ml pooled IgG or 3 \( \mu \)g/ml LPS for 6 h. Cells were lysed, and TF activity was measured. Values represent the mean and SE of two different experiments. The positive control (LPS) confirms that NF-\( \kappa \)B, p38 MAPK, and TLR4 pathways were inhibited by addition of the inhibitors.

A possible confounder of our results would be misallocation of samples between clinical groups. A patient who had only ever suffered PM might nevertheless possess aPLs capable of causing VT and might subsequently develop VT. Therefore, they would be allocated to the VT−/PM+ group, whereas their true allocation should be VT+/PM+. Such misallocations would reduce our ability to detect true differences between the VT+/PM+ and VT−/PM+ groups rather than creating false-positive differences. We feel that allocation of patients to VT+/PM− or VT−/PM+ groups was secure due to the long duration of follow-up (mean = 13 y) without clinical events that would alter that allocation. Of nine female VT+/PM− subjects, five had live births after their VT events, and the other four did not become pregnant for reasons other than APS (e.g., partner’s infertility). Of these nine patients, three are already postmenopausal and six are >40 y, so the chance that they will develop APS-related PM in the future is low.

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FIGURE 6. Effects of pooled VT+/PM+ IgG on monocyte signaling pathways are mediated by anti-\( \beta \)2GPI Abs. Pooled IgG (100 \( \mu \)g/ml) from patients with VT alone (VT+/PM−) was passed through a \( \beta \)2GPI affinity purification column. Eluted fractions were used at a concentration 100\( \mu \)g/ml to treat U937 cells for 6 h. Quantitative analysis displaying ratio of phosphorylated and total protein against NF-\( \kappa \)B.

PM+ subjects might be a mixture of Abs capable of causing VT (with properties similar to VT+/PM− IgG) and those not capable of causing VT (with properties similar to VT−/PM+ IgG). Therefore, the relative amounts of these two types of Ab in each individual VT+/PM+ sample would determine whether it stimulated the p38 MAPK and NF-\( \kappa \)B pathways in our assays. Lastly, the character of the aPL present in an individual might change with time such that the properties of a VT+/PM+ IgG sample would depend on the most recent clinical event in that person. For instance, if the individual had suffered VT long ago but PM recently, then Abs would behave like VT−/PM+ IgG. We believe that our findings of a mixed effect upon these signaling pathways with no correlation with the most recent clinical event support the second of the hypotheses suggested above—that the population of aPLs in these patients is a mixture of aPLs resembling the VT+/PM+ group and aPLs resembling VT−/PM+.

Use of pooled samples was necessary to carry out the multiple experiments to establish the optimal time of incubation with IgG. To reduce the chance of erroneous findings arising from atypical individual samples within pools, we tested two different pools for each clinical group and repeated the experiments using individual samples drawn at random from those used to create each pool. These experiments confirmed the differences between effects of VT+/PM− and VT−/PM+ samples. It is unlikely that these effects are due to non-aPL autoantibodies, because affinity-purified anti-\( \beta \)2GPI from our VT+/PM− group had a particularly strong effect. IgG from the aPL+/APS− group had minimal effect on monocytes in any assay, despite the fact that 9 of 12 patients from this group had SLE and a range of other serum autoantibodies (e.g., anti-dsDNA and anti-Ro).

What is the mechanism of the effects exerted on monocytes by our VT+/PM− IgG samples? We believe that IgG anti-\( \beta \)2GPI Abs within those samples bind to \( \beta \)2GPI (derived from FCS used in cell culture of U937 cells or attached to the monocytes extracted from human serum) and that these complexes interact with TLR4 on the monocyte surfaces. This hypothesis would be consistent with our results showing that the effect of APS IgG upon monocytes is concentrated within the anti-\( \beta \)2GPI fraction of VT+/PM− IgG and inhibited by the TLR4 antagonist E. coli K12 msbB LPS. This antagonist is known to be specific for the TLR4 pathway (from experiments in ECs) and acts upstream of the MyD88 adaptor (29–31). However, aPLs can also act by other mechanisms...
involve other receptors (32). Our results did show some effect of TLR2 blocking Ab, though this was not clear enough to convince us that TLR2 is involved in the effect of our VT+/PM− samples on monocytes. For example, aPLs could be acting by a complement-dependent mechanism, as suggested in other studies (33, 34) because complement is present in the FCS used in our U937 culture medium. Furthermore, we have previously detected β2GPI in IgG purified mononuclear aPLs (24) and have confirmed (data not shown) that copurified β2GPI is also present in our purified polyclonal IgG aPL.

It would be of interest to know whether these differences in effects of IgG samples from patients with different clinical manifestations of APS are mirrored in their effects when tested in in vivo models. Though several such models have been described for both thrombosis (35, 36) and pregnancy loss (37), careful analysis of the methods sections of these papers (both ours and others) shows that very few samples from patients with PM alone have ever been tested in vivo models. Thus, it is currently not possible to reach a conclusion as to whether VT+/PM− and VT−/PM+ IgG aPL samples have different effects in these models.

In conclusion, our data support our hypothesis that aPLs from VT+/PM− and VT−/PM+ patients with APS differ in their effects on p38 MAPK and NF-κB signaling pathways and TF activity. These differences may be mediated by preferential activation of TLR4 by IgG aPL from VT+/PM− patients.

Disclosures
S.S.P. is a co-owner and founder of Louisville APL Diagnostics, Inc. The authors have no other conflicting financial interests.

References

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