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THROMBOSIS AND HEMOSTASIS

Changes in regulation of human monocyte proteins in response to IgG from patients with antiphospholipid syndrome

Vera M. Ripoll,[1] Anastasia Lambrianides,[1] Silvia S. Pierangeli,[2] Katie Poulton,[1] Yiannis Ioannou,[1,3] Wendy E. Heywood,[4] Kevin Mills,[4] David S. Latchman,[5] David A. Isenberg,[1] Anisur Rahman,[1] and Ian P. Giles[1]

[1]Centre for Rheumatology Research, Division of Medicine, University College London and Department of Medicine, Rayne Institute, London, United Kingdom; [2]Division of Rheumatology, Department of Medicine, University of Texas Medical Branch, Galveston, TX; [3]Arthritis Research UK Centre for Adolescent Rheumatology, University College London, London, United Kingdom; [4]Biological Mass Spectrometry Centre University College London Institute of Child Health, London, United Kingdom; and [5]Birkbeck, University of London, London, United Kingdom

Key Points

- Comprehensive proteomics analysis in human monocytes exposed to APS-IgG has identified and characterized several novel proteins.
- These proteins have functional relevance to the APS.

The effects of immunoglobulin G (IgG) from patients with the antiphospholipid syndrome (APS) upon monocyte activation have not been fully characterized. We carried out a comprehensive proteomic analysis of human monocytes treated with IgG from patients with different manifestations of the APS. Using 2-dimensional differential gel electrophoresis (2D DiGE), 4 of the most significantly regulated proteins (vimentin [VIM], zinc finger CCH domain-containing protein 18, CAP Gly domain-containing linker protein 2, and myeloperoxidase) were differentially regulated in monocytes treated with thrombotic or obstetric APS IgG, compared with healthy control (HC) IgG. These findings were confirmed by comparing monocytes isolated from APS patients and HC. Anti-VIM antibodies (AVAs) were significantly increased in 11 of 27 patients (40.7%) with APS. VIM expression on HC monocytes was stimulated more strongly by APS IgG from patients with higher-avidity serum AVA. We further characterized the proteome of thrombotic APS IgG–treated monocytes using a label-free proteomics technique. Of 12 proteins identified with the most confidence, 2 overlapped with 2D DiGE and many possessed immune response, cytoskeletal, coagulation, and signal transduction functions which are all relevant to APS and may therefore provide potential new therapeutic targets of this disease. (*Blood*. 2014;124(25):3808-3816)

Introduction

Pathogenic antiphospholipid antibodies (aPLs) which cause vascular thrombosis (VT) and/or pregnancy morbidity (PM) in patients with the antiphospholipid syndrome (APS) bind β_2-glycoprotein I (β_2GPI).[1] This aPL-β_2GPI interaction in the presence of a second stimulus leads to cellular activation and upregulation of proinflammatory/coagulant factors on target cells,[2] such as tissue factor (TF) on monocytes.[3-5]

Current tests used to identify aPL in patients with the APS are anticardiolipin (aCL) and/or anti-β_2GPI and/or lupus anticoagulant (LA) assays.[6] Positive results, however, in these assays often fail to predict clinical outcomes. For instance, some patients with these aPL will develop only thrombosis whereas others manifest only PM despite prolonged follow-up.[7]

Very few studies have specifically compared effects of samples from patients with and without thrombosis. López-Pedrera et al found differences in p38 mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) signaling pathways as well as TF, vascular endothelial growth factor (VEGF), and proteinase-activated receptors 1 and 2 (PAR1 and 2) expression[4,8,9] in monocytes from APS patients with thrombosis compared with those extracted from patients with nonthrombotic APS and healthy controls (HCs). We purified immunoglobulin G (IgG) from patients with APS who had VT but no PM (VT^+/PM^-) or PM but no thrombosis (VT^-/PM^+). We found that only VT^+/PM^- IgG activated NF-κB, p38MAPK, and upregulated TF activity in monocytes[5] despite there being no significant differences in aPL binding between the VT^+/PM^- and VT^-/PM^+ samples.

Most previous studies have focused on specific cellular pathways when dissecting the mechanism of action of aPL and very few have taken a proteomics approach to identify novel pathways in patients with APS. A proteomic analysis of monocytes isolated from 51 patients with the APS by López-Pedrera and colleagues identified the differential expression of several monocyte proteins between thrombotic and obstetric APS subgroups[10] and found differences in regulation of these proteins by statins.[11]

These studies have used classical, 2-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) followed by mass spectrometry (MS) proteomic techniques to identify novel pathways. Newer techniques such as fluorescence 2D differential gel electrophoresis (2D DiGE) and non-gel-based label-free quantitative methods now exist, allowing more rapid, reproducible, and accurate protein identification and quantitation.

Here, we describe the first experiments using these newer proteomic techniques to further characterize cellular targets and signaling pathways in human monocytes exposed to IgG from patients with APS. We have identified and characterized several novel proteins that have functional relevance to manifestations of the APS.

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Materials and methods

Patients

Serum samples were obtained from 50 individuals for this study with informed consent and appropriate local ethical approval in accordance with the Declaration of Helsinki. Of 27 patients fulfilling APS classification criteria,[6] 11 had systemic lupus erythematosus (SLE) fulfilling classification criteria[12] and 16 had primary APS. The 23 HCs were aPL negative.

Immunological characterization and purification of IgG

IgG was protein G purified, passed through endotoxin removal columns (Thermo Scientific), and confirmed to be <0.06 endotoxin units per mL by *Limulus* amebocyte lysate assay (Sigma-Aldrich). Concentration was determined by spectrophotometry. IgG aCL and anti-β_2GPI titers were determined as previously.[13] Serum LA was measured by dilute Russell viper venom time.

Detection of anti-VIM antibodies

Maxisorp plates were coated overnight at 4°C with 5 μg/mL human recombinant vimentin (VIM) (Sigma-Aldrich) in phosphate-buffered saline (PBS) on the test half and PBS alone on the control half. Plates were washed and blocked for 1 hour at room temperature (RT) with 5% skimmed milk/PBS Tween (PBS-T). After washing with PBS-T, sera (1:50) or IgG in blocking buffer were incubated for 1 hour at RT, with a positive serum calibrator. Bound IgG was detected by anti-human IgG horseradish peroxidase (HRP)–conjugated antibody in blocking buffer for 1 hour at RT followed by addition of substrate for 15 minutes when the reaction was stopped and absorbance read at 405 nm. Net (test minus control) optical density (OD) was expressed in arbitrary units (AUs) in comparison with that of the calibrator IgG and positivity defined as values >3 standard deviations (SDs) above the mean in HC.

To determine anti-VIM antibody (AVA) avidity, chaotropic conditions were introduced. Maxisorp plates were coated overnight at 4°C with 5 μg/mL VIM and blocked. Purified IgG (200 μg/mL) in 5% skimmed milk in PBS-T containing increasing concentrations of NaCl (0.15 M, 0.25 M, 0.35 M, 0.5 M, 1 M, 2 M, 3 M, and 4.5 M) was loaded onto the plate for 1 hour at RT and IgG detected as described in the previous paragraph. Avidity was calculated from the percentage of maximum IgG binding (at 0.15 M NaCl) maintained with each concentration of NaCl.

Isolation and culture of human monocytes

Human promonocytic (U937) cells[14] were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Mononuclear blood cells were isolated from buffy coat residues pooled from 4 healthy donors (National Health Service Blood and Transplant Service) by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation and monocytes purified by Easysep human $CD14^+$ selection (StemCell Technologies). For proteomic analysis, purified monocytes were cultured in serum-free RPMI 1640 medium. For proteomic target validation, monocytes were cultured in RPMI 1640 medium/10% heat-inactivated FBS.

In vitro exposure of monocytes to IgG

For proteomic analysis, 1×10^6 monocytes or U937 cells were treated with 100 μg/mL pooled IgG (containing equal concentrations of individual samples, n = 7) from APS (VT^+/PM^- or VT^-/PM^+) or HC for 6 hours. aPL binding of each pooled IgG sample was tested at 100 μg/mL and VT^+/PM^- and VT^-/PM^+ IgG had high aCL (>40 G phospholipid units [GPLU]) and anti-β_2GPI (>60 standard units [SU]) binding whereas pooled HC IgG lacked aPL binding. For proteomic target validation, 2.5×10^5 monocytes were treated with 200 μg/mL individual: VT^+/PM^- (n = 10), VT^-/PM^+ (n = 8), or HC (n = 10) HC IgG for 6 hours.

Proteomics techniques

2D DIGE, 2D gel electrophoresis, and MS and liquid chromatography (LC)–MS/MS label-free quantitation were performed as described previously.[15-17] Full detail is given in the supplemental Methods (available on the *Blood* Web site).

Data processing and database searching

ProteinLynx GlobalServer version 2.4 was used to process all data acquired. Protein identifications were obtained by searching UniProt/Swiss-Prot databases. Absolute quantitative data in femtomoles and nanograms was analyzed in Microsoft Excel and statistical significance obtained using the Student *t* test.

Validation of proteomic data by qPCR and immunoblotting

Methods regarding monocyte RNA extraction, quantitative real-time PCR (qPCR), and immunoblot are detailed in the supplemental Methods.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software 4.0c (GraphPad Software). Normally distributed data were analyzed by parametric *t* test and nonnormally distributed data by nonparametric tests. In multiple comparisons, statistical analysis was undertaken using 1-way analysis of variance (ANOVA).

Results

Clinical and laboratory characteristics of individuals

A total of 27 patients with APS (19 VT and 8 PM) and 23 HCs subjects were studied (Table 1). Thirty-nine (78%) of the 50 subjects were female. Of 27 patients with APS, 11 had SLE (clinical and serological details are shown in Table 1) and 16 primary APS, similar to larger APS cohorts.[18] Nineteen patients with VT^+/PM^- had thrombotic (12 venous, 11 arterial and 5 recurrent) manifestations. Seven patients with VT-/PM^+ had second-trimester fetal losses and 1 a third-trimester fetal loss. Serum and purified IgG from patients in the VT^+/PM^- and VT^-/PM^+ groups had significantly higher levels of aCL and anti-β_2GPI activity compared with HC, but these levels did not differ significantly between VT^+/PM^- and VT^-/PM^+ groups.

Effects of aPL upon the monocyte proteome

Protein lysates from monocytes treated with pooled IgG from 3 groups, VT^+/PM^-, VT^-/PM^+, and HC, were labeled with Cy Dye DiGE and resolved in 2D gels (supplemental Figure 1). Clear differences in the proteome of monocytes treated with APS IgG compared with HC IgG were observed and the number of proteins differentially regulated by more than twofold in U937 and ex vivo monocytes was then determined (supplemental Table 1). In U937 cells treated with IgG from patients with VT, 52 proteins were differentially regulated compared with HC (41 proteins upregulated and 11 proteins down regulated). In contrast, only 23 proteins (22 of which were upregulated) were differentially expressed in U937 cells treated with PM IgG compared with HC.

The ex vivo monocytes were significantly more responsive to both VT and PM IgG compared with U937 cells. A total of 140 proteins were differentially regulated (119 upregulated and 21 downregulated) after exposure to VT IgG compared with HC IgG, whereas for PM IgG, 127 proteins (105 upregulated and 22 downregulated) were differentially expressed. The pattern of response in U937 cells and ex vivo monocytes was similar in that both VT and PM IgG induced more proteins than they repressed. VT and PM IgG also induced similar sets of proteins in both U937 cells and ex vivo monocytes, respectively. Of the 52 proteins induced by VT in U937s, 24 were

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Table 1. Clinical and laboratory features of patients and controls

	VT, n = 19	PM, n = 8	HC, n = 23
Age, mean ± SEM, y	47.3 ± 3.3	42.1 ± 2.0	38 ± 4.8
Sex	15 F/4 M	8 F	16 F/7 M
PAPS (%)	9 (47.3)	7 (88)	0
SLE (%)	10 (53)	1 (12)	0
Live births	9	13	6
Total APS-related PM	0	8 (7 ST-PL, 1 TT-PL)	0
VT	12 V, 11 A, 5 R	0	0
Other disease features	haem 8, arth 8, muc 7, neuro 6, serositis 4, renal 3	0*	0
Serum LA-positive	13	6	NT
Serum aCL, mean GPLU ± SEM	143.6 ± 19.2	113.0 ± 18.3	9.1 ± 0.8
Serum anti-b_2GPI, mean GDU ± SEM	86.1 ± 19.5	72.3 ± 26.3	12 ± 0.4
IgG aCL, mean GPLU ± SEM	101.7 ± 16.7	76.4 ± 19.8	7.5 ± 0.5
IgG anti-b_2GPI, mean GDU ± SEM	57.1 ± 12.5	64.7 ± 33.3	9.5 ± 0.5
Other autoantibodies	anti-dsDNA 8, anti-Ro 4, anti-La 2, anti-RNP 2	0*	0
Medication	OA 19, CS 5, HCQ 4, AZA 3, LDA 2, MMF 1	LDA 7, HCQ 2*	0

A, arterial; aCL, anti-cardiolipin antibody; anti-β_2GPI, anti-β_2-glycoprotein-I antibody; arth, arthritis; AZA, azathioprine; CS, corticosteroid; dsDNA, double stranded DNA; F, female; GDU, IgG arbitrary unit; GPI, glycoprotein I; GPLU, IgG phospholipid unit; haem, hematological; HCQ, hydroxychloroquine; LDA, low-dose aspirin; M, male; MMF, mycophenolate mofetil; muc, mucocutaneous; neuro, neurological; NT, not tested; OA, oral anticoagulant; PAPS, primary APS; PM, pregnancy morbidity; R, recurrent; RNP, ribonucleoprotein; SEM, standard error of the mean; ST-PL, second-trimester pregnancy loss; TIA, transient ischemic attack; TT-PL, third-trimester pregnancy loss; V venous.

*Incomplete information on SLE of patient with SLE/APS.

also induced by VT in ex vivo monocytes and of the 23 proteins regulated by PM in U937s, 10 were also induced by PM in ex vivo monocytes. Furthermore, there was significant overlap in the proteins induced by both types of aPL. Nineteen proteins were commonly regulated by both VT and PM IgG in U937 cells and 77 proteins in ex vivo monocytes.

Identification and characterization of novel aPL-regulated proteins in monocytes isolated from healthy volunteers

The most significantly regulated proteins with the highest fold change in U937 cells and ex vivo monocytes treated with VT IgG and/or PM IgG were selected for MS analysis. Six protein targets were identified: VIM and zinc finger CCH domain-containing protein 18 (ZC3H18) in both U937 and ex vivo monocytes; POTE ankyrin domain family member E in U937 cells alone; and CAP Gly domain-containing linker protein 2 (CLIP2), myeloperoxidase (MPO) and β-actin-like protein 3 solely in ex vivo monocytes. These proteins have cytoskeletal, nucleic acid binding, and inflammatory related functions (Table 2).

Based upon the 2D DiGE analysis, these target proteins (identified by mW and pI) were all upregulated by both VT and PM IgG compared with HC IgG (Table 2). In U937 cells, VIM was increased by VT IgG (3.8 fold) more than PM IgG (2.3 fold), whereas ZC3H18 (increased 2.9 fold by VT IgG and 2.7 fold by PM IgG) and POTE ankyrin domain family member E (2.7 fold by VT IgG and 2.9 fold by PM IgG) were similarly affected by APS IgG subsets. In ex vivo monocytes, only VT IgG increased VIM (3.3 fold) and β-actin-like protein 3 (2.5 fold) expression and had a greater effect upon CLIP2 protein expression (3.6 fold) compared with PM IgG (2.7 fold). In contrast, PM IgG had a greater effect upon ZC3H18 (increased 4.3 fold by PM IgG and 3.2 fold by VT IgG) and MPO (increased 4.2 fold by PM IgG and 3.9 fold by VT IgG).

Validation of 2D DiGE proteomic data

In validation experiments, individual samples were used to confirm that the effects observed with pooled IgG (pooling required because of the complexity and expense of proteomics experiments) were common to samples from the majority of patients with APS. Ex vivo monocytes were treated with individual VT (n = 10), PM (n = 8), or HC (n = 10) IgG for 6 hours. Levels of messenger RNA (mRNA) were measured by qPCR and protein levels were measured by immunoblot (Figure 1A-H). Consistent with the proteomic data, mRNA expression of VIM, MPO, CLIP2, and ZC3H18 was significantly upregulated in monocytes treated with VT IgG compared with HC IgG (Figure 1A-D), even after removal of outlying values (supplemental Figure 2). Only VIM and MPO mRNA were significantly induced in monocytes treated with PM IgG compared with HC IgG (Figure 1A,D). Conditions for the

Table 2. Monocyte proteins regulated by APS identified by 2D DIGE and MS

Accession	SwissProt entry	Protein description	Function	Molecular mass, kDa	pI	Confidence score	Fold change
U937 cells							
Q86VM9	ZCH18_HUMAN	Zinc finger CCCH domain-containing protein 18	Nucleic acid binding	106.3	8.6	74	2.9 in VT vs HC, 2.7 in PM vs HC
P08670	VIME_HUMAN	VIM	Cytoskeleton	53.6	4.8	185	3.8 in VT vs HC, 2.3 in PM vs HC
Q6S8J3	POTEE_HUMAN	POTE ankyrin domain family member E	Cytoskeleton, actin binding	121.3	5.7	92	2.7 in VT vs HC, 2.9 in PM vs HC
Monocytes							
P08670	VIME_HUMAN	VIM	Cytoskeleton	53.6	4.8	147	3.3 in VT vs HC
Q86VM9	ZCH18_HUMAN	Zinc finger CCCH domain-containing protein 18	Nucleic acid binding	106.3	8.6	138	3.2 in VT vs HC, 4.3 in PM vs HC
Q9UDT6	CLIP2_HUMAN	CAP Gly domain-containing linker protein 2	Cytoskeleton	115.8	6.3	47	3.6 in VT vs HC, 2.7 in PM vs HC
P05164	PERM_HUMAN	MPO	Defense	83.8	9.1	132	3.9 in VT vs HC, 4.2 in PM vs HC
Q9BYX7	ACTBM_HUMAN	β-actin-like protein 3	Cytoskeleton, ATP binding	41.9	5.9	167	2.5 in VT vs HC

Protein IDs were assigned by Waters ProteinLynx Global server software and values above 95% confidence were considered. The score and peptide data are shown.
ATP, adenosine triphosphate; pI, isoelectric point.

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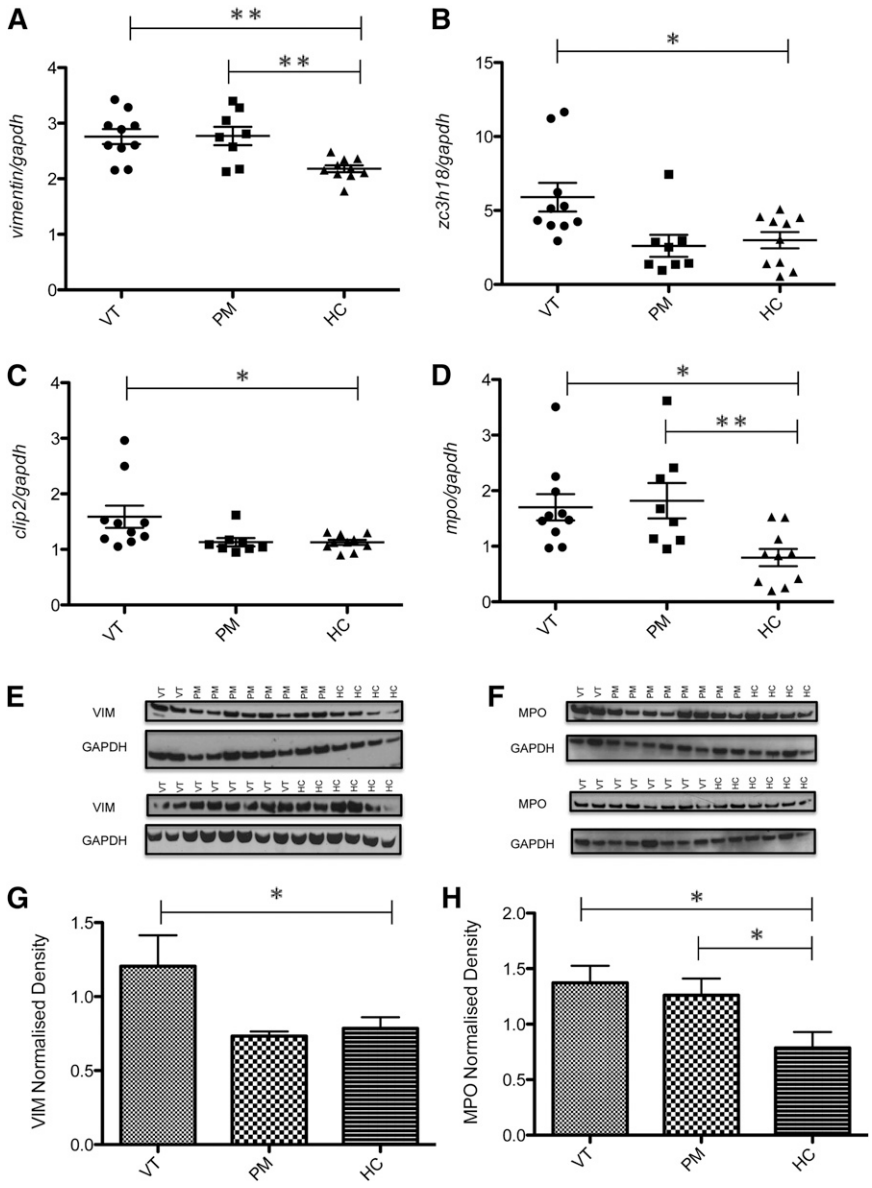

Figure 1. Validation of protein targets identified by 2D DIGE. Ex vivo monocytes were treated with 200 μg/mL of individual IgG samples: 10 VT, 8 PM, or 10 HC for 6 hours and levels of mRNA measured by qPCR (A-D). Data points represent the fold change expression of each sample compared with untreated; mean of duplicates and standard errors are displayed. Data are representative of at least 3 independent experiments. Representative western blots of monocytes treated with the 10 VT, 8 PM, and 10 HC IgG samples are shown (E-F). Blots were scanned and analyzed densitometrically. Graphical representations of the density ratios of each protein and GAPDH expressed in arbitrary scanning units are displayed (G-H). Data are representative of at least 2 independent biological replicates. Statistically significant difference (*$P < .05$, **$P < .005$) was determined by ANOVA. 2D, 2-dimensional; ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative PCR.

POTE ankyrin domain family member E and β-actin-like protein 3 PCR could not be optimized so these proteins were not evaluated.

At the protein level (Figure 1E-H), VIM was significantly upregulated by VT IgG but not PM IgG compared with HC IgG whereas MPO was significantly induced by both VT and PM IgG. Commercial antibodies failed to detect the protein expression of CLIP2 and ZC3H18.

Expression and regulation of proteomic targets in monocytes from patients with APS

We next investigated whether similar effects occur in patients with APS by measuring mRNA expression of VIM, ZC3H18, CLIP2, and MPO in ex vivo monocytes from APS patients with VT (n = 3) and age-matched HC (n = 3) before and after exposure to lipopolysaccharide (LPS) (100 ng/mL) as an inflammatory stimulus. Increased levels of VIM mRNA expression were observed in APS compared with HC monocytes after LPS exposure, whereas basal levels of VIM mRNA were higher in APS but not significantly different between these groups (Figure 2A). Conversely, MPO mRNA expression was significantly higher in untreated monocytes from patients with APS compared with HC and LPS downregulated this response in both APS and HC monocytes (Figure 2B). Similarly to VIM, CLIP2 mRNA expression was only increased in APS monocytes after exposure to LPS (Figure 2C). No significant differences in the expression of ZC3H18 between APS and HC samples were observed either with or without LPS treatment (Figure 2D).

Functional characterization of AVAs in IgG from patients with APS

Given that aCL antibodies/AVAs have been described in APS,[19] we examined whether the induction of VIM in monocytes by APS IgG is associated with the presence of AVAs. IgG AVAs were significantly increased in 11 of 27 patients with APS (40.7%) compared with HC, with no difference in binding between VT and PM IgG (Figure 3A). There was no correlation, however, between the presence of AVA in patients with APS and VIM expression (protein fold change) on ex vivo healthy monocytes exposed to IgG derived from the same patients (Figure 3B).

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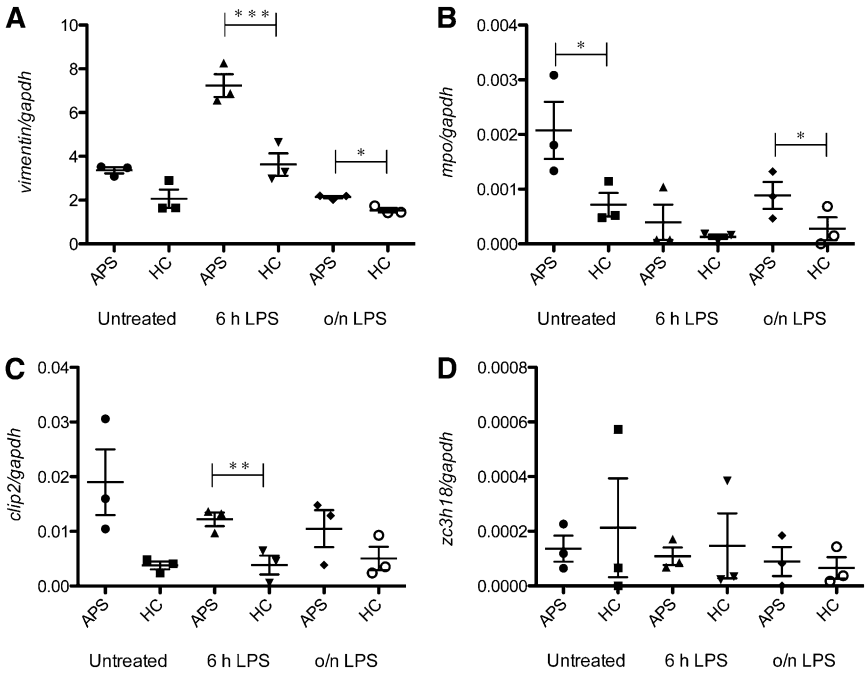

Figure 2. Expression and regulation of proteomic targets in monocytes from patients with APS. Ex vivo monocytes from 3 patients with VT and 3 age-matched HC were left untreated or stimulated with 100 ng/mL LPS for 6 hours or overnight and mRNA levels were measured by qPCR (A-D). Data points represent the mean of duplicates and standard errors are displayed. Statistically significant difference (*$P < .05$, **$P < .005$, ***$P < .001$) was determined by paired Student *t* test. LPS, lipopolysaccharide.

To investigate whether high-avidity AVA correlate with monocyte VIM expression, chaotropic conditions were introduced to the AVA ELISA. IgG samples from 8 patients with sufficient serum for IgG purification were tested and clearly segregated into 2 groups: 4 high- and 4 low-avidity (Figure 3C). APS IgG from the patients with high-avidity AVA stimulated significantly more VIM expression (protein fold change, $P = .0039$) in healthy monocytes than APS IgG from low-avidity patients (Figure 3D).

Identification and quantification of thrombotic-regulated proteins by LC-MS/MS label-free Quantitation

Given that not all proteins are amenable to gel electrophoresis, we fully characterized the proteome of ex vivo monocytes treated with a different set of pooled representative APS-VT or HC IgG (n = 7 in each group) by LC-MS/MS label-free quantitation. We elected to compare only VT and HC IgG because the maximal differences were seen between these groups in previous experiments (Figure 1). A total of 44 proteins were significantly dysregulated; 29 proteins were downregulated and 15 were upregulated (Table 3, supplemental Table 2). The 12 proteins identified with the highest degree of confidence and statistical significance (Table 3) are associated with cytoskeleton, immune response, and coagulant functions. Two of these proteins were also identified by 2D DIGE: POTE ankyrin domain family member E (increased 16.2 fold) and β-actin-like protein 3 (increased 2.4 fold) by VT IgG compared with HC. Other proteins of particular relevance to APS and/or immune response are Coronin 1A (COR1A), plasminogen activator inhibitor 2 (PAI2),

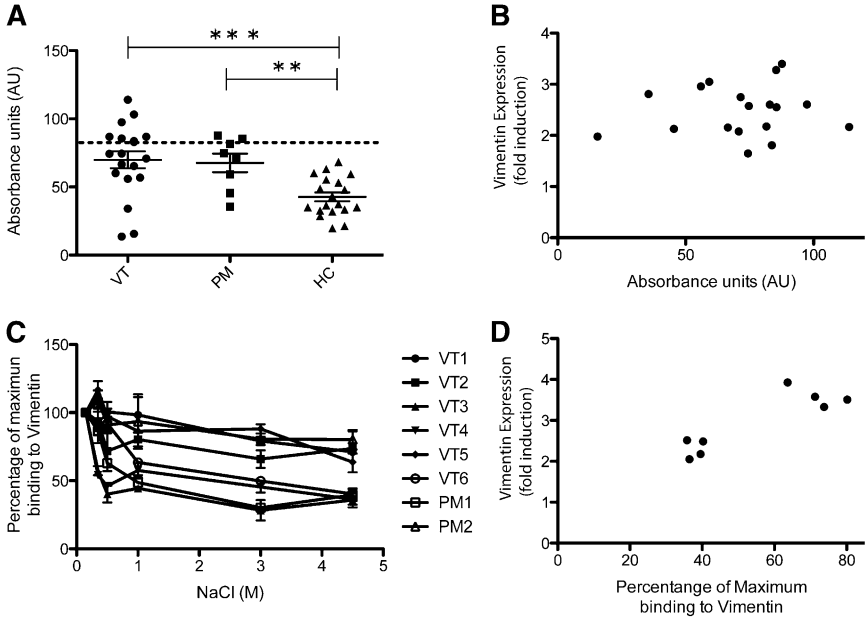

Figure 3. Detection of AVAs in IgG from patients with APS IgG. Levels of AVAs in the IgG of 27 patients with APS (19 VT and 8 PM) and 23 HC were analyzed by ELISA (A). IgG were tested in duplicates at 200 μg/mL, and binding was expressed in AUs in comparison with an in-house positive control. Dashed line depicts the cutoff for positivity, defined as values >3 SD above the mean in HCs (n = 23). Statistically significant difference (**$P < .005$, ***$P < .001$) was determined by ANOVA. The presence of AVA in patients with APS was correlated with VIM expression (protein fold change) on ex vivo healthy monocytes exposed to IgG derived from the same patients (B). Avidity of antibodies for VIM in a subset of APS-positive samples (C). Correlation between the induction of VIM (protein fold change) in monocytes by APS IgG and levels of AVAs (B) or high avidity antibodies ($P = .0039$) (D) is shown. 3D, 3-dimensional; AU, arbitrary unit; ELISA, enzyme-linked immunosorbent assay.

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Table 3. Thrombotic-regulated proteins identified by LC-MS/MS label-free quantitation

Accession	SwissProt entry	Protein description	Function	Confidence score	ANOVA, *P*	Fold change	Regulation
P01857	IGHG1_HUMAN	Ig γ 1 chain C region	Immune response	172.7	.00119	2.3	Up
Q6S8J3	POTEE_HUMAN	POTE ankyrin domain family member E	Cytoskeleton, actin binding	162.8	.03278	16.2	Up
Q93077	H2A1C_HUMAN	Histone H2A type 1 C	DNA binding	131.8	.00348	3.0	Up
P01861	IGHG4_HUMAN	Ig γ 4 chain C region	Immune response	124.4	.00196	2.1	Up
Q96KK5	H2A1H_HUMAN	Histone H2A type ^{1}H	DNA binding	123.1	.00586	3.0	Up
P31146	COR1A_HUMAN	Coronin 1A	Cytoskeleton, actin binding	109.7	.00079	2.7	Down
Q9BYX7	ACTBM_HUMAN	Putative β-actin-like protein 3	Cytoskeleton, actin binding	91.0	.02461	2.4	Up
P01834	IGKC_HUMAN	Ig κ chain C region	Immune response	73.7	.00029	3.0	Up
P05120	PAI2_HUMAN	Plasminogen activator inhibitor 2	Proteolysis, coagulation	73.0	.00006	4.1	Down
Q8IUE6	H2A2B_HUMAN	Histone H2A type 2 B	DNA binding	72.1	.00342	2.6	Up
PO8238	HS90B_HUMAN	Heat shock protein HSP 90 β	Immune response	59.2	.00008	71.0	Down
P31949	S10AB_HUMAN	Protein S100 A11	Immune response, signal transduction	57.4	.04707	3.0	Up

The top 12 monocyte proteins identified with a higher degree of confidence are shown. Protein IDs were assigned by Waters ProteinLynx Global server software and values above 95% confidence were considered. The score and peptide data are shown. *P* values calculated by ANOVA represent differences in the abundance of each protein in the presence of VT or HC IgG. Up and Down indicate the upregulation or downregulation of any given protein in VT IgG compared with HC IgG.

ANOVA, analysis of variance; LC, liquid chromatography; pI, isoelectric point.

and heat shock protein HSP90 (HSP90) which were significantly repressed in the presence of VT IgG whereas S100 (S100 A11) was upregulated by APS antibodies (Table 3).

Validation of LC-MS/MS proteomic data

To validate these proteins ex vivo monocytes were treated with individual IgG, and mRNA expression of COR1A, PAI2, HSP90, and S100 A11 was measured. In accordance with the proteomics data, COR1A and PAI2 expression were significantly downregulated in monocytes treated with VT IgG compared with HC IgG (Figure 4A-B). HSP90 mRNA was repressed by VT IgG compared with HC IgG, but levels did not reach significance and S100A11 expression levels were significantly induced by APS-VT IgG (Figure 4C-D).

Discussion

In this study, we have used different proteomic techniques to compare and characterize multiple proteins simultaneously in Ig-treated monocytes. We identified a number of proteins that are differentially regulated by APS IgG compared with HC IgG.

Classical proteomic techniques measure the intensity of the 2D PAGE protein stain to determine individual protein quantity and confirm its identity by MS. Of the additional techniques we used, 2D DiGE overcomes limitations in traditional 2D electrophoresis due to intergel variation by allowing measurement of the abundance of a protein in each sample by direct comparison with other samples as well as an internal standard (in this case a mix of IgG from all 3 groups) on each gel.

Not all proteins, however, are amenable to 2D DiGE particularly hydrophobic membrane proteins. Therefore, to ensure that we had fully characterized the proteome, we studied tryptic digests of IgG-treated monocyte proteins using LC-MS/MS label-free quantitation on a Q-TOF mass spectrometer. Compared with 2D DiGE, this gel-free technology offers reduced sample consumption, improved detection sensitivity, enhanced data quality, and a reduced influence of intrinsic protein characteristics (such as hydrophobicity) for proteomic studies. The detection, however, of protein isoforms and posttranslational modifications is still complicated using gel-free approaches so it is advantageous to use both 2D DiGE and label-free methods.

Pathway analysis of the novel protein targets that were differentially regulated in our analysis revealed that many of them are involved in immune response, cytoskeletal, coagulation, and signal transduction functions, which are all relevant to APS. In addition, we have identified and validated protein targets found in genomics and classical proteomics APS studies in monocytes and endothelial cells (EC).

Previously, Ortona et al used a proteomic approach to identify autoantigens in EC bound by sera from patients with APS. They identified complexes of VIM with cardiolipin (VIM/cardiolipin [CL]) as a key antigen[19] and showed that anti-VIM/CL antibodies were found in serum from 37 of 40 (92.5%) of patients with APS. VIM is an intermediate filament cytoskeletal protein, which is expressed by a number of different cells undergoing apoptosis or activation such as neutrophils,[20] T cells,[21] monocytes[22] and EC.[23] Furthermore, AVAs have been identified in other autoimmune rheumatic diseases, including SLE[24] and associated with thrombotic manifestations in accelerated graft versus host disease post solid organ transplantation.[25] In this paper, we have extended the findings of Ortona et al showing that VIM may be important in the pathogenesis of APS. In particular, we have shown: presence of AVA (not anti-VIM/CL) in serum of 40.7% of patients with APS; VIM is upregulated at both the protein and mRNA level in healthy monocytes exposed to APS-IgG; VIM mRNA expression is increased in monocytes extracted from patients with APS in the presence and absence of LPS as an inflammatory stimulus; and that high avidity AVA IgG correlates with increased stimulation of monocyte VIM expression.

Several other protein targets we identified have been indirectly linked with APS. POTE ankyrin domain family member E belongs to a family of genes that contain ankyrin repeats. Induction of an ankyrin repeat and BTB (POZ) domain containing gene was identified in an earlier genomics analysis of aPL-induced endothelial cell gene expression.[26] Plasminogen activator inhibitor type-1 (PAI-1) is the main inhibitor of urokinase, PAI-2 is predominantly intra-cellular and has a number of anti-inflammatory, anti-proliferative and anti-apoptotic functions.[27] Therefore, reduced levels of PAI-2 may allow unopposed action of PAI-1 as found in APS[28] to promote thrombogenesis. Similar contrasting actions of PAI-1 and PAI-2 are emerging in cancer biology.[29] S100A11 belongs to the family of S100 proteins involved in cell growth and transcription.[30] These proteins are known to interact with Annexin AI and A2 which were identified in the proteomic studies of López-Pedrera et al.[10,11]

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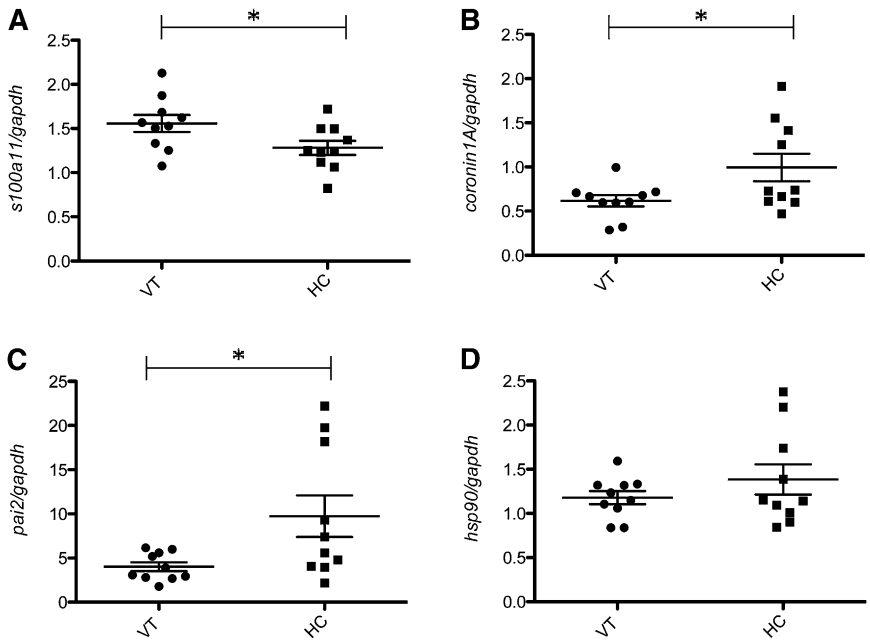

Figure 4. Validation of protein targets identified by LC-MS/MS. Ex vivo monocytes were treated with 200 μg/mL of individual IgG samples (10 VT or 10 HC) for 6 hours and levels of mRNA measured by qPCR (A-D). Data points represent the fold change expression of each sample compared with untreated; mean of duplicates and standard errors are displayed. Data are representative of at least 3 independent experiments. Statistically significant difference ($*P < .05$) was determined by unpaired Student *t* test.

Several other protein targets we identified, have been linked with autoimmune and inflammatory diseases. Levels of MPO (a peroxidase enzyme highly expressed in neutrophils and macrophages) have been associated with development of coronary vascular disease.[31] Anti-MPO antibodies are detected in vasculitides and have been reported in patients with lupus.[32] Coronin is a conserved actin binding protein involved in cytoskeletal function and a single mutation in the Coronin-1A gene has been shown to suppress disease in lupus prone mice.[33] Of the remaining proteins we identified many of them have cytoskeletal function so it is relevant that anti-β_2GPI antibodies mediated induction of endothelial microparticle release is dependent upon phosphorylation of the myosin regulatory light chain and assembly of actin-myosin networks.[34]

Previous proteomics studies on monocytes in APS used classical proteomics techniques to identify dysregulated proteins in ex vivo monocytes from patients with APS.[10,11] López-Pedrera and colleagues identified 22 proteins that were dysregulated in monocytes of 51 patients with APS (31 with thrombosis and 19 with PM alone) compared with HC. Six proteins that were most significantly altered among monocytes from patients with thrombotic APS were all functionally related to the induction of a procoagulant state as well as to autoimmune-related responses and were regulated by statin therapy.[11] Furthermore, proteins implicated in recurrent spontaneous abortion were also determined to be significantly dysregulated in patients with obstetric APS. We identified proteins under similar functional categories; immune response, signal transduction, metabolism as well as a member of the HSP family and catalase. Interestingly, despite clear demonstration of mRNA and protein activity of TF by ourselves and others, neither our own nor other published proteomic/genomic studies in patients with APS identified TF dysregulation. In particular, TF was not found in previous proteomics analysis identifying 22 proteins in ex vivo monocytes isolated from 51 patients with APS[10] or 6 proteins in ex vivo monocytes isolated from 42 patients with APS treated with statins.[11] Furthermore, TF was not identified among 101 genes found to be induced in a genomics analysis of anti-β_2GPI stimulated endothelial cells which are another major source of TF.[26] We hypothesize that this discrepancy between TF gene activation, protein expression and activity may be explained by aPL leading to a cellular re-distribution of intracellular TF to the cell surface as has been shown in monocytes with a high response to LPS.[35] Ultimately, further experiments are required to address this issue.

We do not believe that the inclusion of SLE/APS in our pooled samples has adversely affected our results for several reasons. First, the clinical and serological features of primary APS and SLE associated APS are known to be similar[18,36] and the 2006 APS classification criteria recommended that this distinction be dropped.[6] Second, we have previously shown that whereas IgG from patients with APS (with or without SLE) stimulated phosphorylation of NF-κB or p38MAPK and increased TF activity in human monocytes compared with HC IgG, purified IgG from aPL-positive patients with SLE but no APS (APL^+/APS^-) did not stimulate any of these effects in monocytes.[5] This lack of effect was seen despite the fact that 9 of 12 patients from this aPL^+/APS^- group had a range of other serum autoantibodies, such as anti-dsDNA and anti-Ro.[5] Finally, to confirm that different autoantibody sub-populations in IgG purified from patients with SLE have not influenced our findings in the current study we reanalyzed results shown in Figs 1, 3, and 4 and found no differential effect of SLE/APS-IgG compared with primary APS-IgG (supplemental Figures 3-5).

Interestingly, we found differential regulation of the monocyte proteome by thrombotic and non-thrombotic APS-IgG in our 2D DiGE analysis. Previously, we found only IgG from patients with thrombotic APS activated NF-κB, p38MAPK and caused upregulation of TF activity in cultured monocytes. In contrast, IgG isolated from patients with obstetric APS, aPL-positive patients without APS or HCs did not.[5] Allocation of patients to VT^+/PM^- or VT^-/PM^+ groups was secure due to the long duration of follow-up of at least 10 years, without clinical events that would alter that allocation.

Therefore, we have identified several novel proteins and validated other known targets in monocytes exposed to APS IgG and in ex vivo APS monocytes. Further studies of the biological relevance

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of these findings are now important to examine the proinflammatory or procoagulant effects that dysregulation of these proteins may have. Given that these proteins have functional implications relevant to the pathogenesis of the APS they could provide potential new targets for the treatment of this disease and its various complications.

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Authorship

Contribution: V.M.R. and A.L. designed and performed the research, analyzed the data, and contributed to the writing of the manuscript; S.S.P. recruited patients and was involved in study design; K.P. helped with purification of IgG and qPCR; Y.I. and D.S.L. contributed to study design and critically revised the manuscript; W.E.H. carried out proteomics analysis and critically revised the manuscript; K.M. carried out proteomics experiments and critically revised the manuscript; D.A.I. contributed to study design and patient recruitment and critically revised the manuscript; A.R. contributed to study design, patient recruitment, and data analysis and critically revised the manuscript; I.P.G. conceived the study, designed the experiments, was involved in data analysis, patient recruitment, and characterization, and wrote the manuscript; and all authors read, revised, and approved the content of the final version of the manuscript.

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The current affiliation for Anastasia Lambrianides is The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus.

Silvia S. Pierangeli died in August 2013.

Correspondence: Ian P. Giles, Rayne Institute, Room 411, 5 University St, London, United Kingdom; e-mail: i.giles@ucl.ac.uk.

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