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ORIGINAL ARTICLE

Simvastatin competitively inhibits cellular signaling of lipid-binding antiphospholipid antibodies

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Abstract

Background: Antiphospholipid antibodies (aPLs) cause antiphospholipid syndrome (APS), a disease characterized by thrombotic events and/or pregnancy morbidity. Some clinical studies suggest that statins may beneficially affect the course of APS.

Objectives: In this study, we studied the effect of statins on aPL-induced monocyte activation, aPL-triggered tissue factor (TF) activation, and the underlying cellular mechanisms.

Methods: Cultured monocytes were treated with human monoclonal lipid-binding aPLs or total immunoglobulin G fractions positive for cardiolipin antibodies with or without statins. Expression of TF and tumor necrosis factor α (TNFα) messenger RNA was measured by real-time quantitative polymerase chain reaction. TF activity was determined using clotting assay. The effects of statins on the binding and internalization of aPL were determined by confocal microscopy and flow cytometry.

Results: Simvastatin completely inhibited the induction of TNFα and TF messenger RNA by human lipid-binding aPLs. Simvastatin also prevented TF activation by aPL on the cell surface. These effects were not mediated by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition, but simvastatin and other statins prevented lipid-binding aPLs from binding to their cell-surface target, ie, lysobisphosphatidic acid (LBPA) presented by the endothelial protein C-receptor (EPCR) by displacement of LBPA. Competition experiments indicate that statins displace LBPA from the hydrophobic groove of EPCR. Consequently, aPLs could not induce any downstream cellular effects.

Conclusion: Our data show that statins prevent monocyte activation and TF-triggered coagulation by interfering with binding of lipid-binding aPLs to the EPCR/LBPA complex on the cell surface of monocytes. These findings may help to understand the observed beneficial effects of statins in APS.

KEYWORDS
antiphospholipid syndrome, endothelial protein C receptor, lysobisphosphatidic acid, pleiotropic effects, simvastatin

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1 | INTRODUCTION

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by persistently elevated levels of antiphospholipid antibodies (aPLs), vascular thrombosis, and/or pregnancy morbidity [1]. Currently, treatment of APS is based on anticoagulant therapy either alone or in combination with aspirin. However, according to the Euro-Phospholipid Study [2], approximately 17% of APS patients develop recurrent thrombotic events within the first 5 years and 14% in the second 5 years, despite secondary prophylaxis with aspirin and anticoagulants.

As pro- and anti-inflammatory cytokines are dysregulated in patients with APS [3], immunomodulatory agents such as corticoids, as well as hydroxychloroquine and statins, have been proposed as adjuvant treatments for the prevention of recurrent thrombosis in APS patients [4]. Statins are competitive inhibitors of cholesterol biosynthesis and are widely used in primary and secondary prevention of coronary artery disease. The impact of statins on atherothrombotic risk was initially attributed to their positive effects on lipid profiles. However, statins have beneficial effects beyond cholesterol lowering, collectively called pleiotropic effects [5]. It is assumed that most of the pleiotropic effects of statins are due to inhibition of synthesis of isoprenoids, which serve as lipids anchor for small GTPases and mediate their attachment to the plasma membrane.

Recent reports indicate that patients with APS could also benefit from statin therapy irrespective of their cholesterol-lowering effects by preventing aPL-induced expression of tissue factor (TF) or expression of adhesion molecules such as intercellular adhesion molecule-1 and E-selectin [6,7] on endothelial cells, which are involved in development of atherosclerotic plaques. Statins not only regulate the procoagulant properties of endothelial cells but also of monocytes, which play an important role in the pathogenesis of APS [8,9]. These cell culture- and animal model-based results have also been confirmed in patient studies. In 3 trials comparing the levels of inflammatory markers before and after the use of statins, APS patients receiving fluvastatin exhibited a significant reduction in serum levels of tumor necrosis factor α (TNFα) and soluble TF [3,10,11]. Moreover, retrospective analysis of 184 APS patients treated with statins for dyslipidemia indicated that statin therapy can significantly reduce the risk of recurrent thrombosis [12]. In addition to thrombotic APS, there is evidence that statins positively affect pregnancy outcomes in obstetric APS. In 21 women with obstetric APS, statin use during pregnancy improved placental blood flow and reduced preeclampsia features [13].

In summary, these data indicate that statins could be a promising treatment option for APS patients. Statins are generally very well tolerated and could therefore be an interesting, relatively low-risk alternative to anticoagulant therapy, as already discussed by Brey et al. [14]. Statins may have a favorable profile, after accounting for the risk of bleeding during vitamin K antagonist therapy as well as the costs, the need for frequent blood monitoring, and the numerous interactions with other medications. However, statins may not be effective in all patients with APS due to the heterogeneity of aPLs and the variety of pathomechanisms in APS. Therefore, it is important to precisely understand the effect of statins on the procoagulant and proinflammatory properties of aPLs. Thus, the objective of this study was to examine the underlying mechanisms of statins on upregulation of TF and TNFα messenger RNA (mRNA) expression and TF activation mediated by lipid-binding aPLs on monocytes.

2 | METHODS

2.1 | Materials

Simvastatin in lactone form, pravastatin, rosuvastatin, atorvastatin, and δ-Lacton mevalonic acid were obtained from Sigma-Aldrich. Inactive simvastatin was hydrolyzed to active hydroxyl acid form by adding ethanol and sodium hydroxide. The 18:1 Bis(Monoacylglycero)phosphate (S,R) lysobisphosphatidic acid (LBPA) was obtained from Avanti Polar Lipids.

2.2 | Human monoclonal aPLs

The human monoclonal aPLs HL5B and HL7G were obtained from patients with primary APS and have been described in detail previously [15–20]. Lipid-binding aPL HL5B shows cross-reactivity with phosphatidylserine and cardiolipin but lacks reactivity against β2-glycoprotein-I (β2GPI) and has weak lupus anticoagulant activity. Its cell-surface target is the endosomal phospholipid LPBA presented by the endothelial protein C-receptor (EPCR). The human monoclonal aPL HL7G exhibits dual reactivity, as it can react with EPCR/LBPA or β2GPI. As a negative control, irrelevant human monoclonal immunogloublin (Ig) Gs were used isolated by the same method as HL5B. $F(ab')_2$ fragments of HL5B were generated using a commercial $F(ab')_2$ preparation kit (ThermoFisher). All antibody preparations were tested for the presence of the endotoxin by limulus amoebocyte lysate assay ToxinSensor from GenScript and had <0.1 endotoxin units per milliliter.

2.3 | Affinity purification of total human IgG fractions

Sera from patients positive for anticardiolipin antibodies (aCL) and negative for anti-β2GPI antibodies were applied to protein G spin columns from Thermo Scientific, centrifuged, and washed. Eluted IgG fractions were obtained by applying elution buffer (pH 2.7) and immediately neutralized. Eluted fractions were concentrated and resuspended in phosphate-buffered saline (PBS). aCL and anti-β2GPI levels were determined by QUANTA Flash aCL and anti-β2GPI assays.

2.4 | Mouse monoclonal antibodies

Mouse monoclonal antibodies to mouse EPCR (Mab 1650 and Mab 1682) were previously described in detail [15]. Mab 1682 binds to

EPCR only when the receptor is loaded with the phospholipid LBPA and is therefore called anti-EPCR/LBPA 1682. It prevents cellular signal induction by HL5B and has no intrinsic signaling activity [15]. Mab 1650 binds to EPCR independent of LBPA. It is labeled anti-EPCR 1650. Monoclonal antibodies were produced under endotoxin-free conditions. Both EPCR antibodies were labeled with the fluorescent dye Alexa Fluor 647 using a standard protocol.

2.5 | Monocytic cell line

The human monocytic cell line, MonoMac 1 (MM1, RRID: CVCL_1425), was cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Life Technologies), 1% L-glutamine (Gibco), 1% minimum essential medium nonessential amino acids (Gibco), and 1% sodium pyruvate (Merck), without adding antibiotics in 5% CO_2 at 37 °C. The cell cultures were routinely passaged twice a week and regularly tested for mycoplasma contamination using Mycoplasma PCR Detection Kit (Applichem).

2.6 | Isolation of peripheral blood monocytes

Human monocytes were obtained from peripheral blood mononuclear cells of healthy donors by magnetic cell sorting with CD14 MicroBeads (RRID:AB_2665482; Miltenyi Biotec), resuspended in RPMI 1640 medium with supplements described in section 2.5, and cultured in 5% CO_2 at 37 °C overnight.

2.7 | Isolation of mouse spleen monocytes

Mouse monocytes were isolated from spleens of untreated C57BL/6J wild-type mice using CD115 MicroBead kit (Miltenyi Biotec), resuspended in RPMI 1640 medium with supplements described in section 2.5, and cultured in 5% CO_2 at 37 °C overnight. Mouse experiments were approved according to German regulations by Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany (#23-177-07/G18-1-003S; 23 177-07/ G 23-1-034).

2.8 | Cell stimulation

MM1 cells or primary human monocytes were plated in 24-well cell culture dishes at a density of 1×10^6 cells/mL and incubated overnight. The next day, cells were stimulated with HL5B or control IgG at 37 °C for 3 hours. Simvastatin and TF inhibitors were added 15 minutes before stimulation, unless otherwise specified. Mevalonate was added at time zero of the stimulation, immediately after the addition of aPLs. LBPA (10 μM) was added 30 minutes before addition of antibodies.

2.9 | TNFα ELISA

The concentration of TNFα in the cell culture supernatant was assayed using the human TNFα DuoSet ELISA kit from Bio-Techne according to the manufacturer's instructions. For this purpose, MM1 cells were plated the day before and stimulated with HL5B or total human IgG fractions at 37 °C for 6 hours. Simvastatin and LBPA were added 15 minutes before the stimulation.

2.10 | Confocal laser scanning microscopy

For live cell microscopy, MM1 cells were resuspended in RPMI medium without phenol red and incubated with azide-free Fc Receptor Blocker (Innovex Biosciences) for 30 minutes to avoid nonspecific antibody binding to Fc receptors. Subsequently, the cells were pretreated with simvastatin and LBPA as indicated for 30 minutes and stimulated with monoclonal aPLs HL5B, HL5B $F(ab')_2$ fragments or control IgG (400 ng/mL each) for another 30 minutes. Both monoclonal antibodies were labeled with fluorescein isothiocyanate (FITC) with the FITC Antibody Labeling Kit (Thermo Fisher Scientific). Nuclei were visualized with NucBlue (Thermo Fisher Scientific), and late endosomes/lysosomes were stained with LysoTracker (50 nM, Thermo Fisher Scientific). Cells were imaged directly in chamber slides (Thermo Fisher Scientific) maintained at 37 °C on a Zeiss LSM 710 NLO confocal laser scanning microscope.

2.11 | Clotting assay

MM1 cells (1×10^6 cells/mL) were treated with monoclonal aPL HL5B or control IgG at 37 °C for 15 minutes. Simvastatin (10 nM) was added 15 minutes before stimulation. Thereafter, MM1 cells were washed and resuspended in PBS at a concentration of 2×10^6 cells/mL and mixed with normal prewarmed citrated plasma from healthy volunteers at a ratio of 1:1. Clotting time was measured using a KC-4 coagulometer (Amelung) after recalcification with prewarmed $CaCl_2$ solution (30 mM) and converted into procoagulant activity (PCA). The standard curve for conversion to PCA was created using serial dilutions of recombinant human TF (HemosIL RecomciPlasTin 2G; Werfen) in PBS.

2.12 | Flow cytometry

Primary mouse splenic monocytes were incubated with statins for 15 minutes and stained with directly labeled anti-ECPR/LBPA 1682 antibodies or anti-ECPR 1650 antibodies (20 μg/mL) at 4 °C or at room temperature for 30 minutes. The monocytes were washed 3

TABLE List of primers used for gene expression detection and quantification.

Gene-specific primer	Forward primer	Reverse primer
β-actin	GGCATCCTCACCCTGAAGTA	GGGGTGTTGAAGGTCTCAAA
hTNFα	CTTCTCCTTCCTGATCGTGG	GCTGGTTATCTCTCAGCTCCA
hTNFα	AACATCCAACCTTCCCAAACG	GACCCTAAGCCCCCAATTCTC
hTF	GGGCTGACTTCAATCCATGT	GCTGCCCAGAATAACAATGT

hTNFα, human tissue necrosis factor alpha; hTF, human tissue factor.

times with PBS to remove unbound antibodies. Cell-surface expression of EPCR and EPCR/LBPA complexes were analyzed in a BD FACSLyricFlow Cytometer.

2.13 | Gene expression

TF and TNFα gene expression was determined by real-time quantitative polymerase chain reaction. Total RNA was purified from monocytes using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. The RNA was reverse-transcribed into complementary DNA according to the manufacturer's instructions using the Biozym complementary DNA Synthesis Kit. Relative quantification of TF and TNFα expression was evaluated by real-time polymerase chain reaction on the CFX Connect Real-Time System from Bio-Rad. β-actin served as reference gene. All primers used in this study are listed in the Table below.

2.14 | Statistical analysis

Mean and standard deviations of biological replicates are shown. GraphPad Prism 9.5.0 was used for statistical analysis by one-way analysis of variance (ANOVA), followed by Sidak's multiple comparison test.

3 | RESULTS

3.1 | Simvastatin inhibits aPL-induced TNFα expression in a Mevalonate-independent manner

As aPLs induce the expression of various cytokines, the first objective of this study was to determine whether statins would have an impact on the induction of TNFα mRNA by the human monoclonal aPL HL5B. Treatment of the human monocytic cell line MM1 or primary human monocytes with simvastatin completely suppressed the upregulation of TNFα mRNA induced by HL5B (Figure 1A, C). The same effect of simvastatin on TNFα upregulation was also observed with human monoclonal aPL HL7G, which has dual reactivity for EPCR/LBPA and β2GPI, and IgG fractions from 5 patients positive for aCLs and negative for anti-β2GPI antibodies (Figure 1A, C). Characteristics of sera from these patients are summarized in Figure 2. Determining the TNFα protein levels in the cell culture supernatant confirmed that simvastatin inhibits upregulation of TNFα protein expression and secretion by HL5B and IgG fractions (Figure 2).

By inhibiting mevalonate synthesis, statins also prevent the synthesis of isoprenoids. Two recent studies demonstrated that the inhibitory effect of statins on aPL-induced cell activation could be reversed by adding mevalonate [6,7]. Although the rapid inhibition makes mevalonate depletion as a mechanism unlikely, we tested whether addition of mevalonate could restore aPL responses. As shown in Figure 1B and D, addition of mevalonate did not reverse the inhibitory effects of simvastatin on TNFα expression in MM1 cells (Figure 1B) or human primary monocytes (Figure 1D).

Next, we analyzed the time course of simvastatin action in more detail. TNFα upregulation was completely inhibited even when simvastatin was added briefly (15 seconds) after addition of aPLs (Figure 1E). When simvastatin was added 30 seconds after aPLs, inhibition was only partial, and after 1 minute, no inhibition was achieved. In the initial experiments, we used simvastatin at a concentration that is commonly reported in the literature for *in vitro* studies [21]. However, the mean serum concentrations observed in humans with therapeutic doses of simvastatin are much lower and range from approximately 1 to 15 nM [21]. Therefore, we analyzed if simvastatin was also effective at concentrations achieved in human plasma. Simvastatin at concentrations as low as 9 nM also entirely prevented upregulation of TNFα by aPLs (Figure 1F).

3.2 | Simvastatin prevents cell-surface binding and internalization of lipid-reactive aPLs

Since simvastatin inhibits aPL-induced TNFα expression even when added at the same time or shortly after aPLs, we hypothesized that statins interfere with binding of HL5B to its surface target. Recently, this target has been identified as the endosomal phospholipid LBPA presented on the cell surface by EPCR [15]. Accordingly, we investigated by confocal laser scanning microscopy whether simvastatin interferes with binding of monoclonal lipid-reactive aPL HL5B to EPCR/LBPA. As previously described, HL5B is rapidly internalized into endosomes when incubated with MM1 cells. After 30 minutes, there was complete colocalization of HL5B with the endolysosomal marker LysoTracker (Figure 3). When MM1 cells were treated with simvastatin, HL5B was not observed on the cell surface or within the cell (Figure 3). This indicated that simvastatin blocked binding of

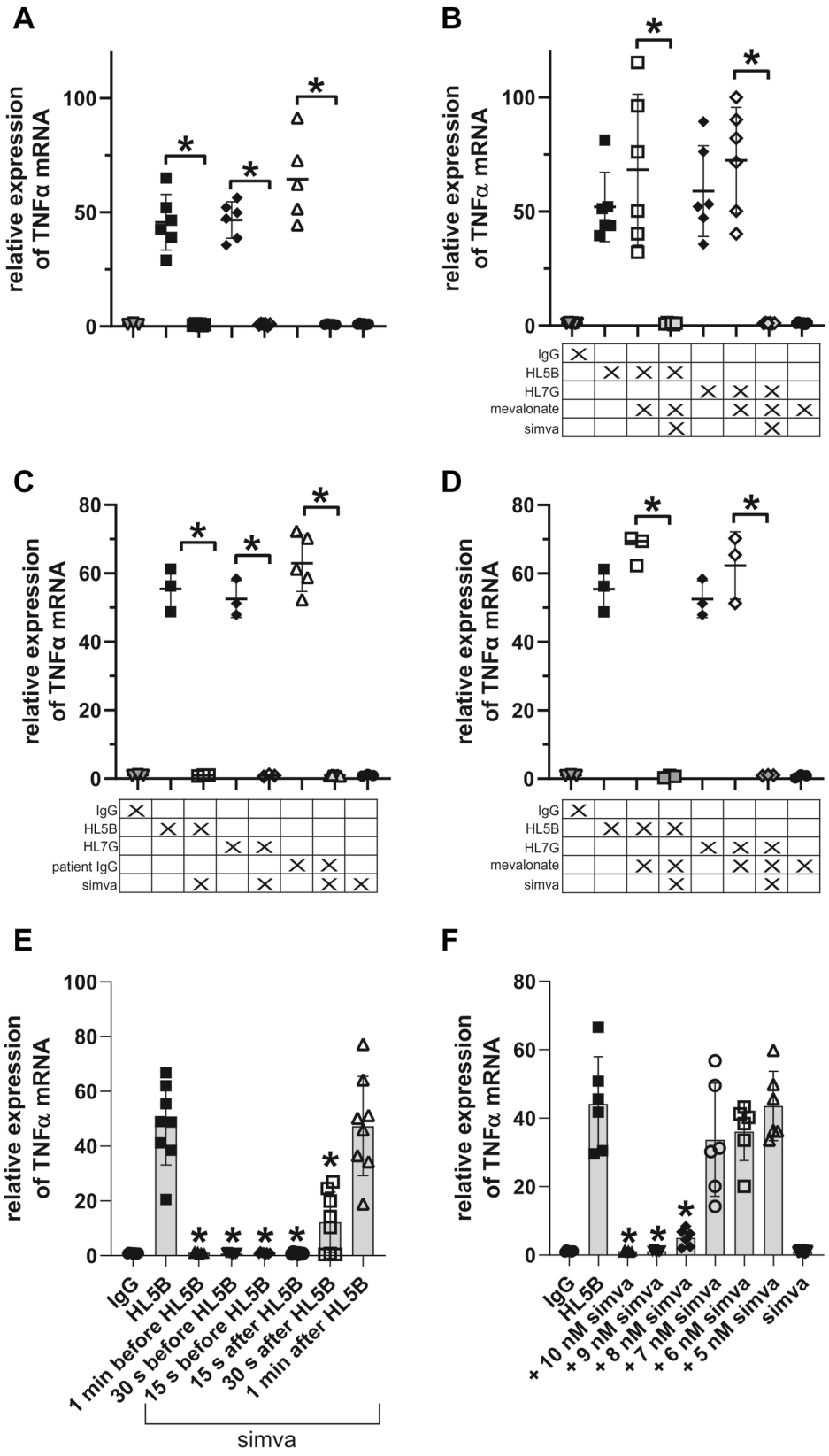

FIGURE 1 Simvastatin at nanomolar concentration immediately inhibits aPL-induced upregulation of TNFα in mevalonate-independent manner. MonoMac 1 (MM1) cells were pretreated with simvastatin (A, 24 μM; B and C, 10 Nm; D: as indicated) for 30 minutes (A, B, D) or as indicated (C). Cells were then stimulated with HL5B or human isotype control IgG (0.4 μg/mL each) either alone or together with mevalonate (6 mM). (A-D) Induction of TNFα mRNA was normalized to unstimulated cells and β-actin expression after 3 hours of stimulation. Data are shown as mean ± SD, n = 3 to 8 independent experiments, $^{**}p < .0001$. aPL, antiphospholipid antibody; IgG, immunoglobulin G; mRNA, messenger RNA; simva, simvastatin; TNFα, tumor necrosis factor alpha.

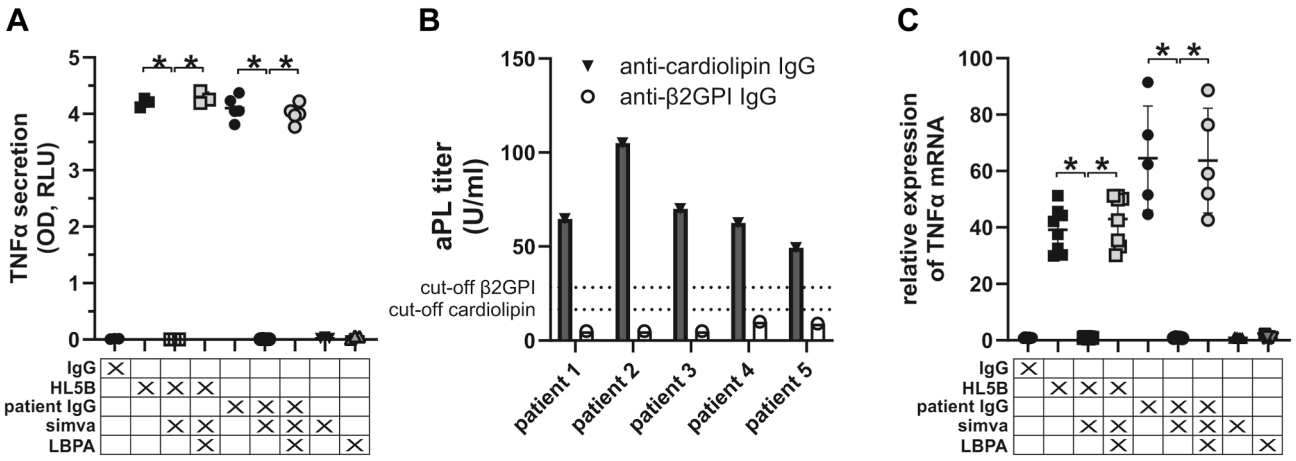

FIGURE 2 Simvastatin blocks binding of HL5B on cell surface and its subsequent internalization Live cell imaging of aPL internalization. MM1 cells were stained for 15 minutes with FITC-labelled HL5B, FITC-labelled F(ab')2 fragments of HL5B or FITC-labelled control IgG (0.4 μg/ml each). HL5B or F(ab')2 stimulated cells were pre-treated with simvastatin (10 nM) with or without LBPA loading (10 μM). Colocalization of internalized aPL and the lysotracker appears yellow. After 15 minutes of incubation, HL5B colocalizes with the endo-/lysosomal marker, while the F(ab')2 fragments of the antibody remains at the cells surface due to the lack of ability to activate the complement system. When simvastatin is added neither internalization of HL5B nor cell surface binding of the whole antibody or its F(ab')2 fragment is observed. The effect of simvastatin is reversed by addition of LBPA. Scale bar = 10 μm. FITC, fluorescein isothiocyanate; LBPA, lysobisphosphatidic acid; simva, simvastatin.

HL5B to EPCR/LBPA on the cell surface rather than the internalized complex. To confirm this, we repeated the experiment using HL5B F $(ab')_2$ fragments instead of whole antibody. HL5B F$(ab')_2$ fragments bind to EPCR/LBPA, but due to their inability to activate complement, they are not internalized [16]. If simvastatin only blocked internalization or induced degradation, surface binding would be observed by confocal laser scanning microscopy. However, in the presence of simvastatin, no surface binding of HL5B F(ab')2 fragments was observed (Figure 3). Thus, statins must block binding to EPCR/LBPA on the cell surface. Notably, addition of excess LBPA over simvastatin to the culture medium restored binding of HL5B F$(ab')_2$ fragments as well as binding and internalization of HL5B IgG (Figure 3). This suggested that simvastatin interfered with LBPA presentation by EPCR, the cell-surface target of lipid-binding aPLs.

We next showed that competition with excess LBPA also restored aPL induction of TNFα in the presence of simvastatin. We treated MM1 cells with simvastatin and LBPA before incubation with lipid-reactive aPLs or IgG fractions and evaluated the expression of TNFα mRNA. As shown in Figure 2, LBPA reversed the inhibitory effect of simvastatin on aPL-induced upregulation of TNFα.

To exclude that statins reduce the abundance of EPCR on the surface of the cells and to confirm specific alterations in LBPA loading, we assessed cell-surface expression of EPCR and EPCR/LBPA by flow cytometry. Because antibodies with these specificities are only available for mouse EPCR, primary monocytes from wild-type mice were pretreated with simvastatin and stained with labeled murine anti-EPCR 1650, which is not affected by the lipid presented by EPCR. Simvastatin had no effect on binding of anti-EPCR 1650 to murine monocytes (Figure 4A). This showed that simvastatin did not affect surface expression of EPCR.

We next stained mouse monocytes with a monoclonal antibody against EPCR/LBPA (anti-EPCR 1682). Simvastatin markedly reduced binding of anti-EPCR 1682 (Figure 4B), indicating interference with antibody binding to EPCR/LBPA. Notably, when simvastatin was removed by 2 washes at 4 °C, binding of anti-EPCR 1682 was not restored immediately (Figure 4C). However, when cells were kept at room temperature after removal of simvastatin, binding of anti-EPCR 1682 was fully restored after approximately 30 minutes (Figure 4E, F). EPCR is known to recycle through the endosomal compartment where it is loaded with LBPA. The data showing that prolonged incubation was needed for restoring anti-EPCR 1682 binding therefore indicated that simvastatin displaced LBPA from the binding groove of EPCR rather than competitively blocked the epitope of anti-EPCR 1682. Notably, pravastatin (Figure 4D) and 2 other statins (atorvastatin and rosuvastatin, data not shown) had the same effects as simvastatin. Simvastatin also showed a clear dose response in inhibiting binding of anti-EPCR 1682 to mouse monocytes (Figure 5A) and HL5B to MM1 cells (Figure 5B).

3.3 | Simvastatin prevents TF upregulation and procoagulant activation induced by lipid-reactive aPLs

We and others have shown that aPLs increase the expression of TF mRNA [22,23]. In addition, we have previously shown that aPLs also rapidly decrypt inactive TF on the cell surface of monocytes and endothelial cells [24]. Therefore, we tested whether simvastatin affected expression and activation of TF in monocytes. Indeed, simvastatin significantly reduced aPL-induced upregulation of TF mRNA (Figure 6A). Addition of excess LBPA prevented the inhibitory effect of simvastatin of TF mRNA expression. We next investigated the PCA of TF expressed on monocytes. Resting monocytes express TF in an inactive (encrypted)

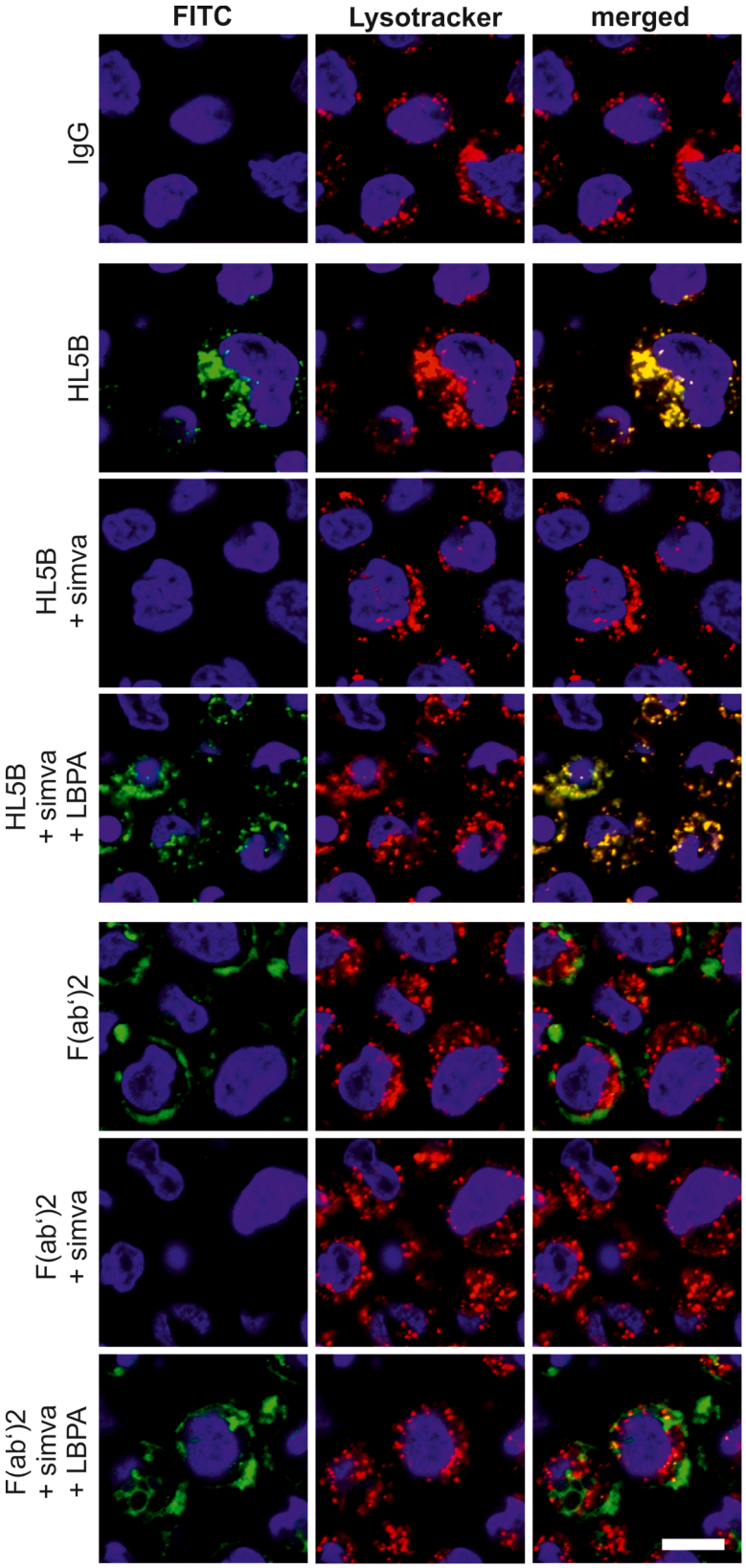

FIGURE 3 LBPA neutralizes the effect of simvastatin on aPL-induced up-regulation of TNFα. MM1 cells were treated with simvastatin (10 nM) and LBPA (10 μM) as indicated for 30 minutes and stimulated with HL5B (0.4 μg/ml), IgG fractions from 5 APS patients (10 μg/ml) or control IgG (10 μg/ml). (A) TNFα levels in the supernatant was measured by ELISA after 6 hours of stimulation. Data are shown as mean ± SD, n = 3 independent experiments, $^{*}p < .0001$; 1-way ANOVA, Dunnett's multiple-comparison test compared with HL5B or patient IgG stimulated cells, respectively. (B) Clinical and laboratory features of the donors used as a source of IgG fractions. Anti-Cardiolipin and anti-β2GPI levels were determined by QuantaFlash Assay (Werfen) with Bioflash. The cut-offs represent 99. percentile. (C) Induction of TNFα mRNA after 3 hours of stimulation was normalized to unstimulated cells and β-actin expression. Data are shown as mean ± SD, n = 3-8, $^{**}p < .0001$; 1-way ANOVA, Dunnett's multiple-comparison test compared with HL5B+simvastatin stimulated cells. LBPA, lysobisphosphatidic acid; simva, simvastatin; β2GPI, β2-glycoprotein 1.

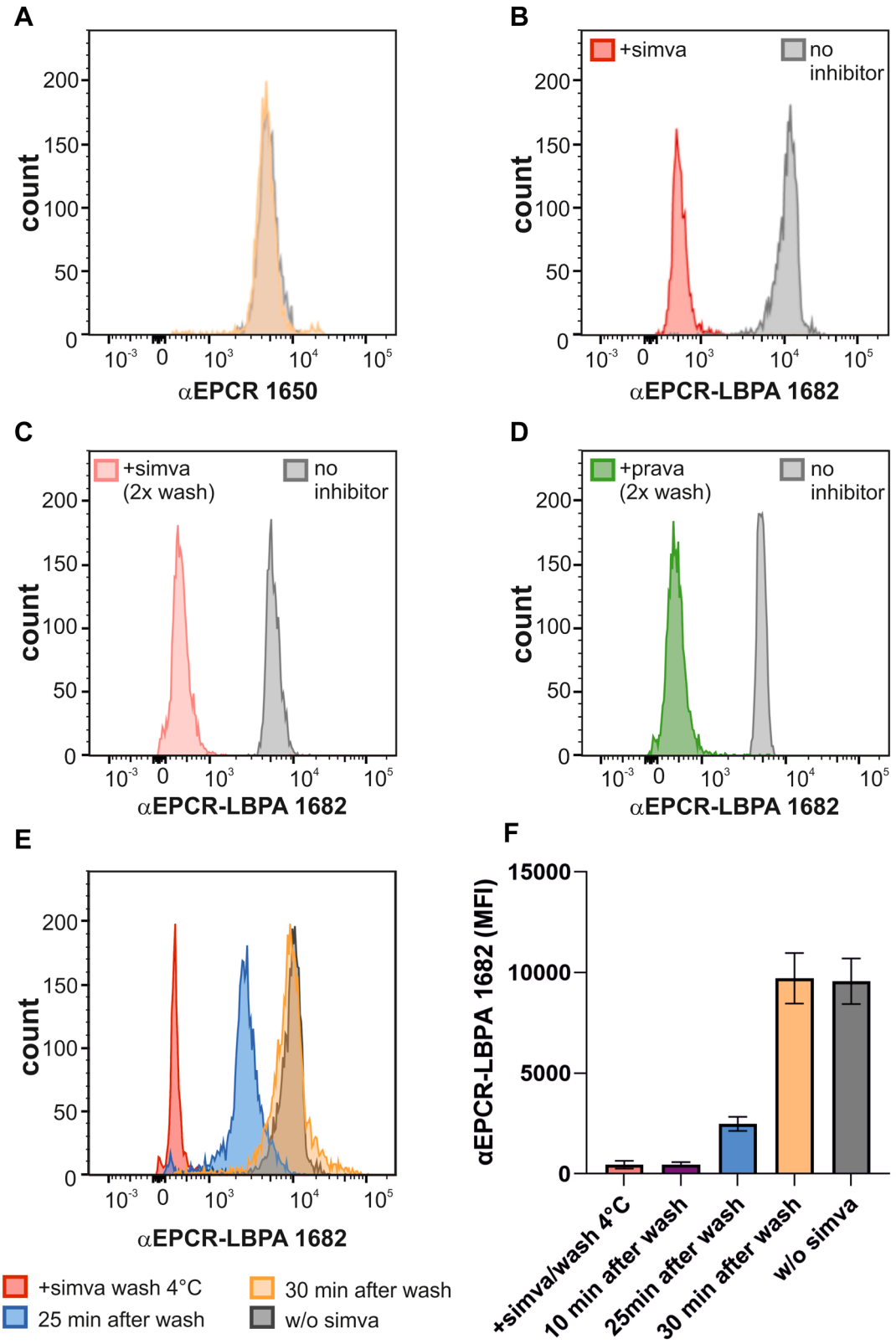

FIGURE 4 Interference of statins with binding of antibody to EPCR/LBPA. Flow cytometric detection anti-EPCR 1650 (A) or anti-EPCR/LBPA 1682 (20 μg/mL each) binding (B-F) to the surface of $CD115^{+}$ spleen monocytes isolated from wild-type mice after treatment with simvastatin or pravastatin (10 nM each). Simvastatin does not reduce binding of anti-EPCR 1650 to murine monocytes (A) but completely prevented binding of or anti-EPCR/LBPA 1682 (B). Removal of simvastatin (C) or pravastatin (D) by 2 washing steps at 4 °C does not restore binding of anti-EPCR/LBPA 1682. However, when cells are returned to room temperature after washing at 4 °C, antibody binding of 1682 could be detected 25 minutes later and is complete at 30 minutes. (E) Representative histogram. (F) Mean fluorescence intensity of anti-EPCR/LBPA 1682 binding. Data are shown as mean ± SD, *n* = 6. EPCR, endothelial protein C-receptor; LBPA, lysobisphosphatidic acid; simva, simvastatin.

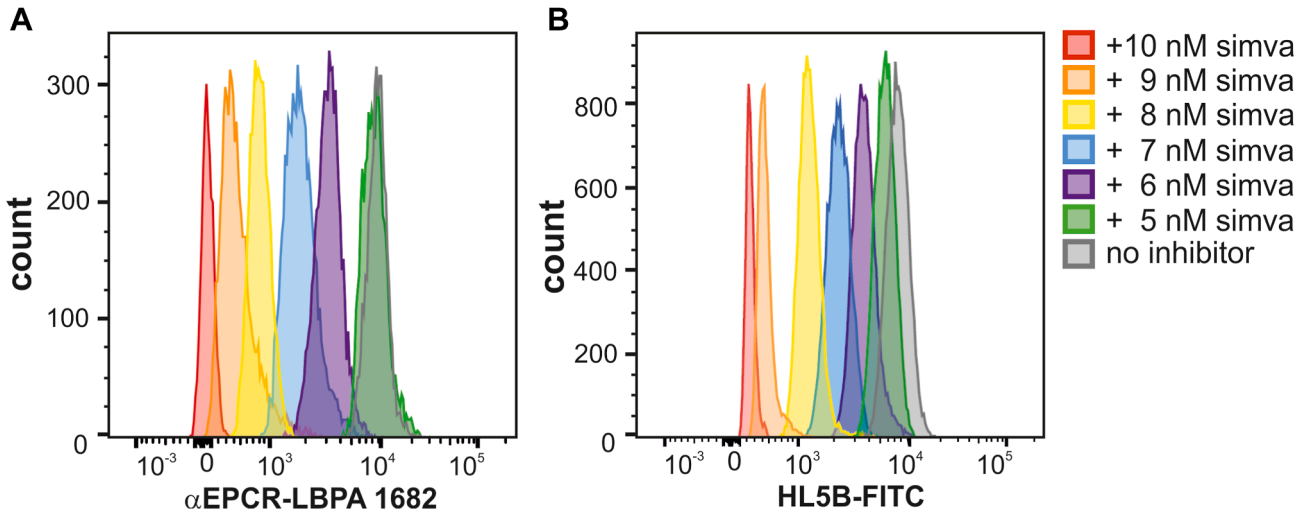

FIGURE 5 Dose-dependent influence of simvastatin on the binding of HL5B and EPCR /LBPA antibodies. Flow cytometric detection of HL5B (A) or anti-EPCR/LBPA 1682 (20 μg/ml each) binding (B) to the surface of MM1 cells (A) or CD115+ spleen monocytes isolated from wildtype mice (B) after treatment with indicated simvastatin concentration. Representative histogram for three independent experiments. EPCR, endothelial protein C-receptor; FITC, fluorescein isothiocyanate; LBPA, lysobisphosphatidic acid; simva, simvastatin.

state complexed with factors VIIa (FXVIIa) and FXa and TF pathway inhibitor (TFPI), which suppresses the initiation of coagulation. After lipid-reactive aPL binds to EPCR, TFPI dissociates from the TF-FVIIa-FXa-TFPI complex [24], and TF is decrypted (activated) in a complement-dependent reaction [16], leading to thrombin generation. As expected, monoclonal aPL HL5B significantly increased the PCA of TF on monocytes. This was prevented by addition of simvastatin and restored with LBPA (Figure 6B).

To confirm the TF dependence of the observed PCA, MM1 cells were treated with monoclonal TF antibodies 10H10, which inhibits TF decryption [24], and 5G9, which blocks substrate binding and TF-initiated coagulation [25]. Both antibodies completely blocked PCA

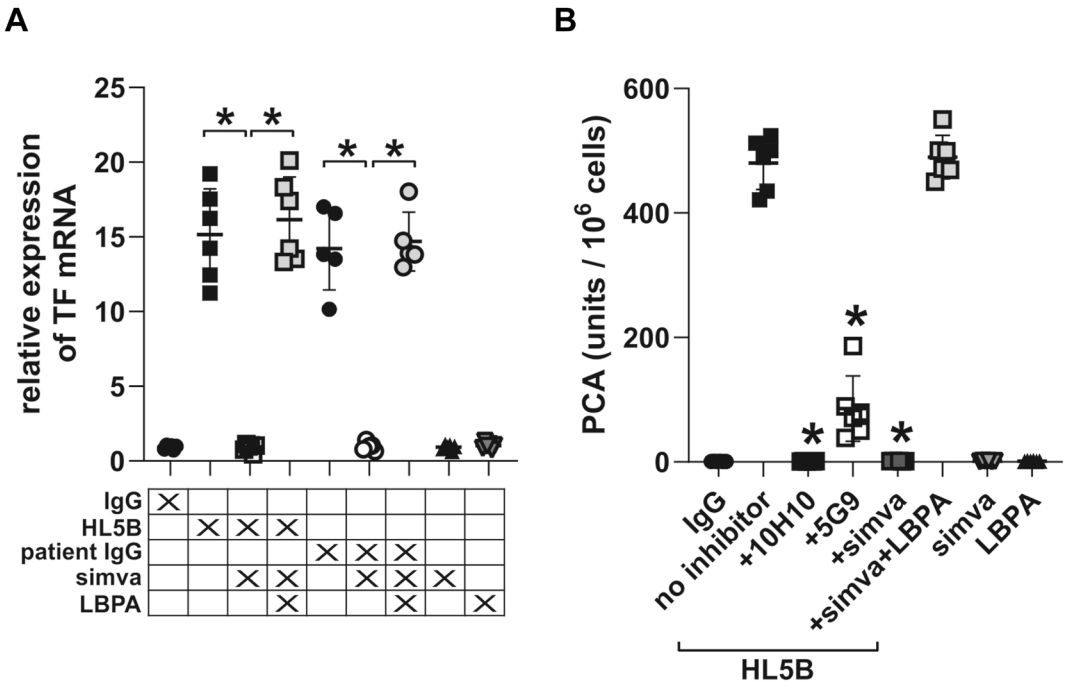

FIGURE 6 The effect of simvastatin of expression of TF. MM1 cells were treated with simvastatin (10 nM) and LBPA (10 μM) as indicated for 30 minutes and stimulated with HL5B (0.4 μg/ml), IgG fractions from 5 APS patients (10 μg/ml) or control IgG (10 μg/ml). Anti-TF antibodies 10H10 or 5G9 (50μg/ml each) were added 15 minutes before HL5B. (A) Induction of TF mRNA after 1 hour of stimulation was normalized to unstimulated cells and b-actin expression. (B) Cell-associated procoagulant activity (PCA) was measured by a single-stage clotting assay. Data are shown as mean ± SD; n = 6; *$p < .0001$; 1-way ANOVA, Dunnett's multiple-comparison test compared with HL5B stimulated cells. LBPA, lysobisphosphatidic acid; simva, simvastatin.

following stimulation with aPL HL5B (Figure 6B), confirming that the procoagulant activity of the activated cells was indeed TF dependent.

4 | DISCUSSION

The present findings confirm that statins can inhibit proinflammatory and prothrombotic effects of lipid-binding aPLs. Recent studies have suggested that statins inhibit aPL-induced increased expression of inflammatory mediators in a mevalonate-dependent manner and that blocking of isoprenoid synthesis by statins may be responsible for these effects [6,7]. Here, we show that statins can also inhibit the effects of aPLs in a mevalonate-independent way by interfering with the cell-surface target of lipid-binding aPLs, ie, LBPA, presented by EPCR.

We have previously shown that the human monoclonal aPL HL5B as well as lipid-binding aPLs isolated from APS patients bind to LBPA presented on the cell surface by EPCR [15]. This is followed by induction of a complex signaling cascade and uptake of aPLs into the endolysosomal compartment [26]. Cellular responses include, among other proinflammatory mediators, the transcriptional induction of TNFα and TF [17,24,27] as well as the rapid activation of cell-surface TF [16,25]. Here, we demonstrate that these effects can be completely inhibited by treatment with simvastatin. Surprisingly, when primary monocytes or MM1 cells are stimulated with aPLs, inhibition of aPL effects by simvastatin is immediate. This time course of simvastatin action indicates that the inhibitory effect of simvastatin is unrelated to the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase and depletion of mevalonate and isoprenoids, which requires several hours or even days to become effective. Accordingly, we find that mevalonate does not reverse the inhibitory effect of simvastatin.

Confocal microscopy demonstrated that simvastatin inhibits binding of lipid-binding aPLs to their surface antigen on monocytes and all further downstream events. Since we have shown previously that signaling of lipid-binding aPLs depends on their binding to the endosomal phospholipid LBPA presented on the cell surface by EPCR [15], it is not surprising that inhibition of this interaction completely abolishes all signaling effects of lipid-binding aPLs. By flow cytometry (Figure 4A), we show that statins do not affect surface expression of EPCR as binding of anti-EPCR 1650 to mouse monocytes is unaltered. However, simvastatin and 3 other statins interfere with binding of anti-EPCR 1682, a monoclonal antibody to the mouse EPCR/LBPA complex. When statins are removed by washing at 4 °C, binding of anti-EPCR 1682 cannot be restored. However, extended recovery of the cells at room temperature for 30 minutes is sufficient to fully restore binding of anti-EPCR 1682 to cell-surface EPCR. Since EPCR cycles from the cell surface to the endosome and returns to the cell surface, these observations suggest that statins displace LPBA from cell-surface EPCR, and through receptor recycling, EPCR is loaded with LBPA again and serves as target for lipid-binding aPLs. Indeed, coincubation of cells with statins and LBPA indicates that the process of LPBA displacement from EPCR is reversible and can be overcome by excessive LBPA.

Pleiotropic effects of statins are typically considered to result from a deficiency of isoprenoid precursor mevalonate. However, Weitz-Schmidt et al. [28] showed that statins can selectively bind to a novel allosteric binding site on β2 integrin function-associated antigen-1 and thus reduce adhesion and costimulation of lymphocytes. Notably, this effect is common to different statins, in agreement with our data, which show that several statins interfere with the interaction of lipid-binding aPLs with EPCR/LBPA. Thus, targeting EPCR/LBPA is another example of a mevalonate-independent pleiotropic effect of statins. Such effects of statins may be overlooked when cells are incubated with statins for extended periods. For example, Stein et al. [29] observed that fluvastatin reduced chemotaxis of vascular smooth muscle cells by a mevalonate-independent mechanism after 20 minutes of incubation to the same extent as by a mevalonate-dependent mechanism after 20 hours of incubation. Similarly, Ghalali et al. [30] demonstrated that statins act in a mevalonate-dependent or mevalonate-independent manner depending on the incubation time and cell type.

Statin concentrations used in cell experiments are often several-fold higher than maximal therapeutic serum concentration achieved in humans [21]. *In vitro* concentrations of statins are commonly in the low micromolar range (1-50 μM), whereas the mean concentration in human serum is 3 orders of magnitude lower, ie, between 1 and 15 nM for standard-dose therapy. The maximal concentration may reach 50 nM, but this concentration is present in human serum only for a few minutes. In contrast, cell cultures are incubated with statins for several hours to days. Therefore, some authors have questioned the clinical relevance of pleiotropic effects of statins observed *in vitro*. We demonstrate that simvastatin completely inhibits the upregulation of TNFα expression induced by aPLs in monocytes at a concentration of $\geq$ 9 nM, suggesting that clinical therapy with simvastatin may also inhibit the effects of lipid-reactive aPLs *in vivo*.

As lipid-binding aPLs target EPCR/LBPA and induce inflammation, thrombosis, and fetal loss in animal models [15], preventing the interaction between aPLs and EPCR/LBPA by simvastatin may explain, at least partially, the observed reduction in inflammatory markers, decreased thrombotic risk, and lower rates of obstetric complications in APS patients treated with statins [10–13]. Because of their broad range of antithrombotic and anti-inflammatory effects, statins have been proposed as adjunctive therapy for hypercholesterolemic patients with APS and APS patients with recurrent thrombosis [31]. Our findings may justify the use of statins for the prevention and treatment of thrombosis in APS irrespective of cholesterol levels. Interestingly, in 2021, the US Food and Drug Administration relaxed the contraindications for statins in pregnancy [32] since the teratogenic effects of statins observed in animal studies have not been confirmed in humans. Thus, according to the current recommendation, statins may be used during pregnancy after a careful risk-benefit assessment and may be considered an additional option for prevention of obstetric APS.

In conclusion, we show that statins inhibit cellular effects of lipid-binding aPLs by interfering with aPL binding to their cell-surface target EPCR/LBPA. This novel pharmacologic mechanism may

explain several observations in animal models as well as in APS patients. Further work is required to elucidate statin effects on the other major group of aPLs reactive with lipid-binding proteins, ie, anti-β2GPI.

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AUTHOR CONTRIBUTIONS

D.M. designed and performed experiments, analyzed data and prepared the manuscript; P.F., A.H., and S.S. performed experiments; W. R. interpreted experiments and wrote the manuscript; K.J.L. designed experiments, interpreted data and wrote the manuscript; N.M.C. designed and supervised the experiments, analyzed data, and wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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REFERENCES

[1] Schreiber K, Sciascia S, de Groot PG, Devreese K, Jacobsen S, Ruiz-Irastorza G, Salmon JE, Shoenfeld Y, Shovman O, Hunt BJ. Antiphospholipid syndrome. *Nat Rev Dis Primers*. 2018;4:17103. https://doi.org/10.1038/nrdp.2017.103

[2] Cervera R, Serrano R, Pons-Estel GJ, Ceberio-Hualde L, Shoenfeld Y, de Ramón E, Buonaiuto V, Jacobsen S, Zeher MM, Tarr T, Tincani A, Taglietti M, Theodossiades G, Nomikou E, Galeazzi M, Bellisai F, Meroni PL, Derksen RH, de Groot PG, Baleva M, et al. Morbidity and mortality in the antiphospholipid syndrome during a 10-year period: a multicentre prospective study of 1000 patients. *Ann Rheum Dis*. 2015;74:1011–8.

[3] Erkan D, Willis R, Murthy VL, Basra G, Vega J, Ruiz-Limón P, Carrera AL, Papalardo E, Martínez-Martínez LA, González EB, Pierangeli SS. A prospective open-label pilot study of fluvastatin on proinflammatory and prothrombotic biomarkers in antiphospholipid antibody positive patients. *Ann Rheum Dis*. 2014;73:1176–80.

[4] de Jesús GR, Benson AE, Chighizola CB, Sciascia S, Branch DW. 16th international congress on antiphospholipid antibodies task force report on obstetric antiphospholipid syndrome. *Lupus*. 2020;29:1601–15.

[5] Liao JK, Laufs U. Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol*. 2005;45:89–118.

[6] Meroni PL, Raschi E, Testoni C, Tincani A, Balestrieri G, Molteni R, Khamashta MA, Tremoli E, Camera M. Statins prevent endothelial cell activation induced by antiphospholipid (anti–β2-glycoprotein I) antibodies: effect on the proadhesive and proinflammatory phenotype. *Arthritis Rheum*. 2001;44:2870–8.

[7] Ferrara DE, Swerlick R, Casper K, Meroni PL, Vega-Ostertag ME, Harris EN, Pierangeli SS. Fluvastatin inhibits up-regulation of tissue factor expression by antiphospholipid antibodies on endothelial cells. *J Thromb Haemost*. 2004;2:1558–63.

[8] Bruni F, Puccetti L, Pasqui AL, Pastorelli M, Bova G, Cercignani M, Palazzuoli A, Leo A, Auteri A. Different effect induced by treatment with several statins on monocyte tissue factor expression in hypercholesterolemic subjects. *Clin Exp Med*. 2003;3:45–53.

[9] Owens AP 3rd, Passam FH, Antoniak S, Marshall SM, McDaniel AL, Rudel L, Williams JC, Hubbard BK, Dutton JA, Wang J, Tobias PS, Curtiss LK, Daugherty A, Kirchhofer D, Luyendyk JP, Moriarty PM, Nagarajan S, Furie BC, Furie B, Johns DG, et al. Monocyte tissue factor-dependent activation of coagulation in hypercholesterolemic mice and monkeys is inhibited by simvastatin. *J Clin Invest*. 2012;122:558–68.

[10] López-Pedrera C, Ruiz-Limón P, Aguirre M, Barbarroja N, Pérez-Sánchez C, Buendía P, Buendía P, Rodriguez-García IC, Rodriguez-Ariza A, Collantes-Estevez E, Velasco F, Khasmahta M, Cuadrado MJ. Global effects of fluvastatin on the prothrombotic status of patients with antiphospholipid syndrome. *Ann Rheum Dis*. 2011;70:675–82.

[11] Jajoria P, Murthy V, Papalardo E, Romay-Penabad Z, Gleason C, Pierangeli SS. Statins for the treatment of antiphospholipid syndrome? *Ann N Y Acad Sci*. 2009;1173:736–45.

[12] Kwon OC, Park YB, Park MC. Effect of statins on the prevention of recurrent thrombosis in thrombotic antiphospholipid syndrome. *Rheumatology (Oxford)*. 2021;61:1548–55.

[13] Lefkou E, Mamopoulos A, Dagklis T, Vosnakis C, Rousso D, Girardi G. Pravastatin improves pregnancy outcomes in obstetric antiphospholipid syndrome refractory to antithrombotic therapy. *J Clin Invest*. 2016;126:2933–40.

[14] Brey RL. New treatment option for the antiphospholipid antibody syndrome? More pleiotropic effects of the statin drugs. *J Thromb Haemost*. 2004;2:1556–7.

[15] Müller-Calleja N, Hollerbach A, Royce J, Ritter S, Pedrosa D, Madhusudhan T, Teifel S, Meineck M, Häuser F, Canisius A, Nguyen TS, Braun J, Bruns K, Etzold A, Zechner U, Strand S, Radsak M, Strand D, Gu JM, Weinmann-Menke J, et al. Lipid presentation by the protein C receptor links coagulation with autoimmunity. *Science*. 2021;371:eabc0956. https://doi.org/10.1126/science.abc0956

[16] Müller-Calleja N, Ritter S, Hollerbach A, Falter T, Lackner KJ, Ruf W. Complement C5 but not C3 is expendable for tissue factor activation by cofactor-independent antiphospholipid antibodies. *Blood Adv*. 2018;2:979–86.

[17] Müller-Calleja N, Manukyan D, Canisius A, Strand D, Lackner KJ. Hydroxychloroquine inhibits proinflammatory signalling pathways by targeting endosomal NADPH oxidase. *Ann Rheum Dis*. 2017;76:891–7.

[18] von Landenberg C, Lackner KJ, von Landenberg P, Lang B, Schmitz G. Isolation and characterization of two human monoclonal anti-phospholipid IgG from patients with autoimmune disease. *J Autoimmun*. 1999;13:215–23.

[19] Prinz N, Häuser F, Lorenz M, Lackner KJ, von Landenberg P. Structural and functional characterization of a human IgG monoclonal antiphospholipid antibody. *Immunobiology*. 2011;216:145–51.

[20] Müller-Calleja N, Grunz K, Nguyen TS, Posma J, Pedrosa D, Meineck M, Hollerbach A, Braun J, Muth S, Schild H, Saar K, Hübner N, Krishnaswamy S, Royce J, Teyton L, Lemmermann N, Weinmann-Menke J, Lackner KJ, Ruf W. Targeting the tissue factor coagulation initiation complex prevents antiphospholipid antibody development. *Blood*. 2024;143:1167–80.

[21] Björkhem-Bergman L, Lindh JD, Bergman P. What is a relevant statin concentration in cell experiments claiming pleiotropic effects? *Br J Clin Pharmacol*. 2011;72:164–5.

[22] Amengual O, Atsumi T, Khamashta MA, Hughes GR. The role of the tissue factor pathway in the hypercoagulable state in patients

with the antiphospholipid syndrome. *Thromb Haemost*. 1998;79:276–81.

[23] Prinz N, Clemens N, Canisius A, Lackner KJ. Endosomal NADPH-oxidase is critical for induction of the tissue factor gene in monocytes and endothelial cells. Lessons from the antiphospholipid syndrome. *Thromb Haemost*. 2013;109:525–31.

[24] Müller-Calleja N, Hollerbach A, Ritter S, Pedrosa DG, Strand D, Graf C, Reinhardt C, Strand S, Poncelet P, Griffin JH, Lackner KJ, Ruf W. Tissue factor pathway inhibitor primes monocytes for antiphospholipid antibody-induced thrombosis. *Blood*. 2019;134:1119–31.

[25] Versteeg HH, Schaffner F, Kerver M, Petersen HH, Ahamed J, Felding-Habermann B, Takada Y, Mueller BM, Ruf W. Inhibition of tissue factor signaling suppresses tumor growth. *Blood*. 2008;111:190–9.

[26] Prinz N, Clemens N, Strand D, Putz I, Lorenz M, Daiber A, Stein P, Degreif A, Radsak M, Schild H, Bauer S, von Landenberg P, Lackner KJ. Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. *Blood*. 2011;118:2322–32.

[27] Müller-Calleja N, Ruf W, Lackner KJ. Lipid-binding antiphospholipid antibodies: significance for pathophysiology and diagnosis of the antiphospholipid syndrome. *Crit Rev Clin Lab Sci*. 2024;61:370–87.

[28] Weitz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J, Bruns C, Cottens S, Takada Y, Hommel U. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat Med*. 2001;7:687–92.

[29] Stein JJ, Maier KG, Gahtan V. Abstract 488: Acute fluvastatin pleiotropy is mevalonate independent. *Arterioscler Thromb Vasc Biol*. 2012;32:A488.

[30] Ghalali A, Wiklund F, Zheng H, Stenius U, Högberg J. Atorvastatin prevents ATP-driven invasiveness via P2X7 and EHBP1 signaling in PTEN-expressing prostate cancer cells. *Carcinogenesis*. 2014;35:1547–55.

[31] Cohen H, Cuadrado MJ, Erkan D, Duarte-Garcia A, Isenberg DA, Knight JS, Ortel TL, Rahman A, Salmon JE, Tektonidou MG, Williams DJ, Willis R, Woller SC, Andrade D. 16th international congress on antiphospholipid antibodies task force report on antiphospholipid syndrome treatment trends. *Lupus*. 2020;29:1571–93.

[32] US Food and Drug Administration. FDA requests removal of strongest warning against using cholesterol-lowering statins during pregnancy; still advises most pregnant patients should stop taking statins. https://www.fda.gov/drugs/drug-safety-and-availability/fda-requests-removal-strongest-warning-against-using-cholesterol-lowering-statins-during-pregnancy; 2021 [accessed July 7, 2025].