Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies


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This work was supported by a grant from the Swiss National Fonds n°310030–127639, by the Dr Henri Dubois-Ferrière-Dinu Lipatti Foundation, and by a grant from the ISTH2007 Presidential Fund.

Short Title: aPLA stimulate monocytes and endothelial cells via TLR2.

Key Words: antiphospholipid antibodies, TLR2, CD14, endothelial cells, monocytes.

Scientific category: Thrombosis and Hemostasis

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Abstract

The presence of antiphospholipid antibodies (aPLA) is associated with arterial or venous thrombosis and/or recurrent fetal loss. Proposed pathogenic mechanisms for aPLA effects include the inflammatory activation of monocytes and endothelial cells and Toll-like (TLR) receptors are candidate signaling intermediates. The aim of this study was to investigate the relative contribution of TLR2 and TLR4 in cell activation by aPLA. Of thirty-two patient-derived aPLA, nineteen induced an inflammatory activation of human monocytes and endothelial cells (HUVEC). In HUVEC, inflammatory responses to these aPLA were increased by TNF-pretreatment, which increases expression of TLR2, but not TLR4. Anti-TLR2 but not anti-TLR4 antibodies reduced aPLA-induced activation of monocytes and HUVEC. aPLA activated TLR2-expressing human embryonic kidney 293 (HEK293) cells but not TLR4-expressing cells. Binding studies demonstrated an interaction between aPLA and TLR2, but not TLR4. A role for CD14, a co-receptor for TLR2 and TLR4, can be inferred by observations that anti-CD14 antibodies reduced responses to aPLA in monocytes and that responses in HEK293 cells expressing TLR2 and CD14 were greater than in HEK293 cells expressing TLR2 alone. Our results demonstrate a role for TLR2 and CD14 in human endothelial cell and monocyte activation by aPLA.
Introduction

The antiphospholipid antibody syndrome (APS) is characterized by clinical manifestations such as arterial or venous thromboembolism and/or recurrent pregnancy complications, as well as the presence of antiphospholipid antibodies (aPLA). Endothelial cells (EC), monocytes and platelets are targeted by the aPLA and inflammatory activation of these cells has been proposed as a pathogenic mechanism. APLA are constituted of heterogenous auto-antibodies that recognize plasma proteins bound to phospholipid surfaces. One antigenic target of aPLA is the plasma phospholipid binding protein \(\beta^2\)-glycoprotein 1 (\(\beta^2\)GP1), which has been detected at the surface of EC and monocytes. Patients with persistent aPLA present only occasional thrombotic episodes, and, sometimes, bacterial or viral infections are associated with the occurrence of clinical manifestations. These observations underline that the presence of aPLA alone is not sufficient to promote thrombosis. Most likely, a «priming factor» of infectious or inflammatory origin is needed. The implication of infection is particularly obvious in catastrophic APS, a rare but fatal subset of APS, which presents characteristic features comparable to those occurring in septic shock.

Stimulation of EC by aPLA was shown to be mediated by intracellular pathways dependent on NF-kB, p38-MAP kinase, MyD88 and TRAF6. The latter mediate signaling by members of the TLR family. The TLRs are a family of integral membrane proteins that recognize conserved pathogen-associated molecular patterns. These receptors thereby function as the first line of defense against pathogens and are essential factors in the innate immune response. Among the ten TLR present in humans, TLR4/MD2 forms homodimers and recognizes lipopolysaccharide (LPS), whereas TLR2 heterodimerizes with TLR1 or TLR6 and recognizes bacterial triacylated or diacylated lipopeptides. TLRs work with accessory proteins, which help in ligand recognition and binding. One of these, CD14, functions as accessory protein for both TLR4 and TLR2. Previously, we have demonstrated that TLR2 is required for activation of mouse embryonic fibroblasts by aPLA. In contrast, in a mouse thrombosis model, the absence of functional TLR4 reduced the prothrombotic effect of aPLA. Monocytes constitutively express TLR2, TLR4 and CD14 whereas EC express TLR4 and very low levels of TLR2 and CD14. Expression of TLR2 by human EC is strongly increased after activation by inflammatory stimuli such as TNF, LPS or interleukin (IL) 1\(\beta\).

The principal aim of the present work was to investigate the respective role of TLR2 and TLR4 in human cell activation by aPLA. We used three cell types for our studies: monocytes,
EC and HEK293 cells expressing either TLR2 or TLR4. Cell activation by aPLA was inhibited by antibodies to TLR2 or CD14, but not by antibodies to TLR4. Binding studies revealed interactions of aPLA with TLR2 but not TLR4. Taken together, these results demonstrate that TLR2 is required for cell stimulation by some aPLA and that CD14 enhances this response.
Materials and Methods

Reagents
Human TNF was from BioGene (Kimbolton, UK); TLR4grade™ LPS from Escherichia coli R515 from Alexis Corporation (Lausen, Switzerland); lipoteichoic acid (LTA) from Staphylococcus aureus and monoclonal anti-human TLR2 blocking antibody (clone TL2.5) were from InvivoGen (San Diego, CA). Anti-human CD14 blocking antibody (clone M5E2), isotype matched control IgG2a and IgG1, Alexa Fluor (AF) 647-conjugated mouse anti-human TLR2 (clone TL2.1), AF647-conjugated isotype matched control IgG2a and anti-human CD32 antibody were from Biolegend (San Diego, CA). Monoclonal anti-human TLR4 blocking antibody (clone 7E3) was from Hycult Biotech (Uden, the Netherlands). AF647-conjugated mouse anti-human TLR4 (clone HTA125), FITC-conjugated mouse anti-human CD14 and FITC-conjugated isotype matched control IgG1 were from AbD-Serotec (MorphoSys AG, Martinsried, Germany). Rabbit polyclonal anti-TLR4 (ab47839) and rabbit polyclonal anti-TLR2 (ab47840) antibodies were from Abcam (Cambridge, UK). Rabbit control IgG (sc-2027) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture
Monocytes- Monocytes were isolated from blood buffy coats of healthy volunteers and provided by the Geneva Hospital blood transfusion center as previously described. Monocyte purity routinely consisted of >90% CD14+ cells, <1% CD3+ cells, and <1% CD19+ cells as assessed by flow cytometry. Cells were cultured in RPMI (Gibco BRL-Life Technologies) containing 10% FBS.

HEK293 cells- Human embryonic kidney 293 (HEK293) cells stably transfected with human TLR4, MD2 and CD14 (HEK-Blue-4) or with human TLR2 and CD14 (HEK-Blue-2) and HEK293 cells stably transfected with human TLR2 (HEK-TLR2) or human TLR4 (HEK-TLR4) were obtained from InvivoGen (San Diego, CA) and grown in Dulbecco’s Modified Eagle’s Medium containing 10% Fetal Bovine Serum (FBS) (Gibco BRL-Life Technologies, Rockville, MD). The HEK-Blue cells express the soluble alkaline phosphatase (sAP) reporter gene under control of the NF-κB promotor, which enables to quantify cell activation by measuring sAP activity in media containing specific enzyme substrate.

Endothelial cells- Human umbilical cords were obtained with written consent from a parent and approval by the institutional ethics committee of the University Hospital of Geneva, in accordance with the Helsinki declaration. EC (HUVEC) were isolated from umbilical cord veins
as described previously. Cells were cultured in EGM-2 medium (Lonza, Walkersville, MD) and used at passage 1.

**Patient characteristics**

Thirty-two patients with antiphospholipid antibodies and clinical manifestations were selected at the Hemostasis Unit of the University Hospital of Geneva. Thirty-one had an antiphospholipid antibody syndrome, as defined by the revised Sapporo criteria. Three of them had associated systemic lupus erythematosus (SLE). One patient had SLE and aPLA. A group of nineteen healthy controls was included. Ten ml of blood was obtained from each patient or control with written consent and approval by the institutional ethics committee of the University Hospital of Geneva, in accordance with the Helsinki declaration.

**IgG purification**

IgG fractions (aPLA) were isolated from patient plasma on Protein-G CL-4B Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). IgG was eluted with 0.1M glycine pH 2.5 and immediately neutralized by addition of 1/4 volume of 1M Tris pH 9.0. Protein levels were measured by the bicinchoninic acid protein assay (BCA Protein assay; Pierce, Rockford, IL). Negative control antibodies were isolated from 19 healthy donor plasmas (CTL). β2GP1-immunopurified aPLA was prepared by passing aPLA through an affinity column made by immobilizing 1 mg β2GP1 per ml of Affigel-HZ (Biorad, Hercules, CA). The bound IgG was eluted with 0.1 M glycine pH 2.5 and immediately neutralized. Endotoxin levels were measured by the Limulus Amebocyte Lysate Endochrome Assay (Charles River Laboratories, Charleston, SC), and found to be below the detection limit (0.25 EU/ml) for all IgG fractions at the concentration used in the assays. To exclude lipopeptide contamination of the IgG fractions, we depleted IgG from the aPLA fractions by one step of affinity adsorption to Protein G-Sepharose and tested the remaining supernatant for HEK-Blue-2 activation (cell-based assay developed by InvivoGen). We found that IgG depletion, evaluated by BCA, resulted in a decreased HEK-Blue-2 activation (see supplementary Figure 1). From these results, we conclude that the cell activation potential of the aPLA preparations was IgG associated.

**Analysis of cell activation**

Different approaches were used to assess activation of the various cell types by the different agonists or antibody preparations.
- **HEK cell activation** – For measuring cell activation by the alkaline phosphatase assay, HEK-Blue-4 or HEK-Blue-2 cells were plated at $4 \times 10^4$ cells/well and incubated for 16h with LPS (100 ng/ml), LTA (0.5 µg/ml), or 500 µg/ml of aPLA or control IgG in HEK-Blue™ Detection Medium (InvivoGen), which contains a specific sAP substrate. Cell activation was assessed by measuring the absorbance change of the detection medium at 650 nm.

For measuring cell activation by analysis of IL-8 secretion, HEK-Blue-2 and HEK-TLR2 cells were seeded in a 96 wells plate at $4 \times 10^4$ cells/well. After 48h, the medium was changed and the cells were incubated for 16h with various concentrations of LTA, aPLA or control IgG. The inflammatory chemokine IL-8 was quantified in cell culture supernatants using the human IL-8 ELISA Kit from R&D Systems (Minneapolis, MN). The HEK-Blue-2 and HEK-TLR2 cell density was determined using the Cell Titer 96®AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI).

- **HUVEC activation** - 2.5 x $10^4$ cells/well were plated in 96 wells plate. Half of the cells were maintained in EGM-2 medium (resting HUVEC), the other half were prestimulated with 100 ng/ml of TNF for 24h followed by an 8h period of TNF wash-out. Then resting HUVEC and TNF-pretreated HUVEC were incubated for 4h with 500 µg/ml of aPLA or control IgG or 10 µg/ml LTA in EGM-2 medium supplemented with 10% FBS. In some experiments, blocking anti-TLR2 or anti-TLR4 antibodies (10 µg/ml) were added to the TNF-pretreated HUVEC 30 min prior the incubation with the stimuli. Endothelial cell activation was assessed by measuring mRNA levels of the leukocyte adhesion molecule E-selectin.$^{24}$

- **Monocyte activation** - Monocytes were seeded at $5 \times 10^5$ cells/well in a 48 wells plate. Blocking antibodies to TLR2, TLR4 or CD14 were added at the concentration of 10 µg/ml for 30 min prior to incubation with 1 µg/ml of LTA, 0.1 µg/ml of LPS, 500 µg/ml of aPLA or control IgG for 4h. The supernatant was collected for TNF quantification by ELISA (R&D System).

Tissue Factor activity was quantified on monocytes (1 x $10^5$ cells/wells in 96 wells plates) after 6 h of stimulation with aPLA or control IgG (500 µg/ml) or β2GP1-immunopurified IgG (100 µg/ml) in presence or absence of blocking antibodies to TLR2, TLR4 or CD14. TF activity was performed as described previously,$^{26}$ using 150 nM FX (Diagnostica Stago, Asnière, France), 5 nM FVIIa (Novo Nordisk Pharma SA, Kusnacht, Switzerland), 1 mM CaCl₂, and 3.5 mM of chromogenic substrate for FXa (Hyphen BioMed, Neuville sur Oise, France). Reading was performed at OD 405nm with kinetic mode. The linear absorbance changes were converted to a concentration of generated FXa by reference to a standard curve made with known amount of FXa (Hyphen BioMed).
Reverse transcription and quantitative real-time PCR (qPCR)

qPCR was done on a StepOne™ Real-Time PCR instrument (Applied Biosystems, Foster City, CA). For tissue factor (TF) mRNA quantification on monocytes, RNA was extracted using the RNeasy® Mini kit (Qiagen, Hilden, Germany), reverse transcribed using the ImPromII™ Reverse Transcription system (Promega, Madison, WI). The TF Taqman probe (Hs00175225_m1) and Taqman master mix reagent were then used. Human GAPDH mRNA served as a control gene for the amount of cDNA present in each sample. Quantification of E-selectin mRNA in HUVEC was done using the Taqman® Gene expression Cell-to-CT™ kit (Ambion, Austin, TX), the Taqman probes for E-selectin gene expression (Hs00950407_m1) and human GAPDH gene expression were used with Taqman® Gene expression Master Mix. Data were analyzed using the comparative ΔCT method and Applied Biosystems StepOne software, according to the manufacturer’s instructions.

Quantification of aPLA binding to TLR2 and TLR4 on HEK-TLR2 or HEK-TLR4, on HUVEC and on monocytes.

The in situ Proximity Ligation Assay 27 with the DuoLink® kit (Olink Bioscience, Uppsala, Sweden) was used to quantify the interaction of aPLA or control IgG with TLR2 or TLR4 on the cell surface. HUVEC and HEK-293 cells (HEK-TLR2 and HEK-TLR4) were grown on Lab-teck® II Chamber Slides (Nalgen Nunc International, Rochester, NY) pre-coated with 0.1% gelatin (Bioconcept, Allschwil, Switzerland) or 15 µg/ml of human fibronectin (R&D Systems) respectively. For HUVEC, half of the wells were incubated with 100 ng/ml of TNF for 24h. Monocytes were seeded at 5 x 10^4 cells per well and allowed to adhere on Lab-tek®II chamber slides. The cells were fixed with 4% paraformaldehyde solution for 5 min, rinsed with PBS and incubated for 1 h in blocking buffer provided by the DuoLink® kit. APLA (P1) or control IgG (C0) at 100 µg/ml or β2GP1-immunopurified IgG at 50 µg/ml were incubated for 1h followed by 3 washes with Tris (10 mM) - NaCl (150 mM), pH 7.5 (TBS) containing 0.05% Tween-20. For monocytes, FCγRII receptors were blocked by incubating cells with 0.5 µg/well of mouse anti-human CD32 antibodies for 10 min prior to the addition of aPLA or control IgG. Rabbit anti-TLR2, anti-TLR4 or control rabbit IgG were incubated at 10 µg/ml for 1 h followed by 3 washes. The slides were then incubated for 1h with oligo-labeled anti-rabbit IgG and anti-human IgG, followed by hybridization, ligation, amplification and detection using a fluorescent probe, according to the manufacturer's instructions (Duolink Detection kit 563, Olink Bioscience). The slides were mounted using Vectashield® mounting medium (Vector Laboratories, Inc.,
Burlingame, CA). The stained cells were analysed with an LSM510 Meta Confocal microscope (Carl Zeiss AG, Germany). We used a x40 objective (EC Plan Neofluar x40 1.3 Oil DIC, Zeiss) for all images. The images were collected using the LSM 510 Flex scan package 2.5 and the AxioVision LE v4.5 softwares (Zeiss). Quantification of the fluorescent surface per cell, identified by Hoechst 33342 blue nuclear staining, was performed using the MetaMorph® v6.0 software (Visitron Systems GmbH, Germany). Results are expressed in arbitrary units, in which the experimental condition with highest fluorescent surface per cell was arbitrarily placed at 100 AU.

**Statistical analysis**

Data are expressed as mean ± standard error (SE) values and analyzed by a two-way ANOVA test followed by a paired t-test or by the Mann-Whitney test using GraphPad Prism version 5.0.
Results

Monocyte activation by aPLA

We investigated to what extent aPLA were capable of activating human monocytes. In this study isolated monocytes were used on the day of isolation, without attachment to plastic. Freshly isolated monocytes were chosen because they express higher levels of TLR2, TLR4 and CD14 than monocytes obtained by adhesion to plastic (Supplementary Figure 2A). Isolated monocytes showed specific responses to LTA (a TLR2 agonist) and LPS (a TLR4 agonist) as demonstrated by the inhibition of TNF secretion and levels of tissue factor mRNA and activity, when blocking antibodies to TLR2 or TLR4 are used (Supplementary Figure 2B). Blocking antibody to CD14 weakly reduced monocyte responses to LTA and LPS at the agonist concentration used in the test. We then investigated activation of monocytes by aPLA. We first established a cut-off value for monocyte activation, as assessed by measuring TNF concentrations in 4h conditioned media, by treating the cells with 19 control IgG's at 500µg/ml. The cut-off value was defined as the average value + 3 SD of the TNF protein results obtained with these control IgG's. Under our experimental conditions the cut-off value was 0.44 ng/ml. We tested 32 aPLA preparations for their ability to activate monocyte and observed that 19 of the latter induced a cell response above the cut-off value (Figure 1A). There was no significant association between cell activation ability and patient characteristics or positivity in any particular aPLA assay (Table 1).

Contribution of TLR2, TLR4 and CD14 to monocyte activation by aPLA

We investigated the role of TLR2 and TLR4 in monocyte activation by the 19 active aPLA preparations using specific blocking antibodies to TLR2 or TLR4. Monocyte activation was evaluated by quantification of TNF secretion and of TF mRNA levels after 4h of stimulation, which correspond to a maximal TF mRNA response to LTA, LPS and aPLA (data not shown). TF activity was determined after 6h of stimulation. With the anti-TLR2 antibodies we obtained a significant inhibition of monocyte TNF protein and TF mRNA responses (p<0.001, two way ANOVA and paired t-test) to the 19 tested aPLA preparations, whereas the anti-TLR4 antibodies (p = 0.7) had no effect (Figure 1B and C). Our observation that blocking antibodies to CD14 significantly reduced (p<0.001) the responses to aPLA implies a role for this protein in monocyte activation by these autoantibodies. Similar results were obtained for TF activity (Figure 1D).

We also investigated the effect on monocytes of β2GP1-immunopurified IgG from two patients and whether the monocyte responses were reduced by preincubation with anti-TLR2 and anti-
TLR4 antibodies. The monocyte response to β2GP1-immunopurified IgG was reduced 58% by anti-TLR2 antibodies whereas anti-TLR4 antibodies or isotype-matched control antibodies (not shown) had no significant effect (Figure 1B-D). Blocking antibodies to CD14 reduced the monocyte responses to aPLA by 57%.

**Cell activation by aPLA in TLR2 or TLR4 reporter gene models**

In the previous section we observed that antibodies to TLR2 had a much stronger inhibitory effect on aPLA-induced monocyte activation than antibodies to TLR4. As these antibodies were developed to block responses to known TLR2 and TLR4 agonists, we cannot exclude the possibility that the blocking anti-TLR2 or anti-TLR4 antibodies are not optimal as inhibitors of cellular responses to aPLA. To further clarify the role of each TLR we took advantage of a well-characterized model system using HEK293 cells stably expressing TLR2 or TLR4 and MD2 (HEK-Blue-2 and HEK-Blue-4 cells) in combination with CD14.

First, we made a detailed comparison of TLR2, TLR4 and CD14 expression by these cells, as well as their responses to LTA or LPS. Flow cytometry analysis revealed that HEK-Blue-2 cells expressed TLR2, but not TLR4, whereas HEK-Blue-4 cells expressed TLR4 but not TLR2 (Supplementary Figure 3A). CD14 was highly expressed in both cell lines, with a similar average fluorescence intensity (Supplementary Figure 3A). Likewise, TLR2 mRNA was only detected in HEK-Blue-2 cells and TLR4 mRNA only in HEK-Blue-4 cells; expression of CD14 mRNA was identical in the two cell lines (Supplementary Figure 3B). HEK-Blue-2 cells responded to LTA with an increased release of sAP, whereas LPS had no effect. In contrast, HEK-Blue-4 cells responded to LPS, but not to LTA (Supplementary Figure 3C). Taken together these results indicate that the two cell lines contain all the signalling intermediates required for inflammatory responses to TLR ligands and only differ in their expression of TLR2 or TLR4/MD2.

We tested the effect of 32 aPLA preparations on HEK-Blue-2 cells and HEK-Blue-4 cells. We observed that the 19 aPLA preparations that induced an inflammatory activation of monocytes also gave a positive response in HEK-Blue-2 cells, whereas the other aPLA preparations induced the same sAP activity as the 19 control IgG preparations. In contrast, none of the aPLA preparations induced a response in HEK-Blue-4 cells (Figure 2). The difference in response of HEK-Blue-2 and HEK-Blue-4 cells to the 19 monocyte activating aPLA was highly significant (p<0.0001; two way ANOVA and paired t-test).
Role of CD14 in TLR2 dependent HEK293 activation by aPLA

In monocytes we observed that inhibition of CD14 reduced the response to aPLA (Figure 1B). To determine whether CD14 also contributes to HEK-Blue-2 activation, we compared responses to aPLA of HEK-Blue-2 cells and HEK293 cells transfected with TLR2 alone (HEK-TLR2) by quantification of IL-8 release. The 19 cell activating aPLA preparations induced a significantly (p<0.001, two way ANOVA and paired t-test) higher response in HEK-Blue-2 cells than in HEK-TLR2 cells; similar results were obtained with aPLA IgG immunopurified on immobilized β2GP1 (p<0.01 (Figure 3A). We used different concentrations of LTA and three different aPLA preparations. The results show that the presence of CD14 shifted the dose-response to lower concentrations but had no effect on maximal responses (Figure 3 B and C).

Contribution of TLR2 and TLR4 on human endothelial cell activation by aPLA

As EC are likely to contribute to the pathological effects of aPLA,28,29 we determined the role of TLR2 in aPLA-induced activation of human EC. We used E-selectin mRNA as a very sensitive marker for inflammatory HUVEC activation. Treatment for 4h of these cells with the 19 cell-activating aPLA induced a small but significant increase in E-selectin mRNA as compared to control IgG (p < 0.001; Mann Whitney test) (Figure 4B). Incubation of the cells with immunopurified anti-β2GP1 antibodies from two different patients also gave a small increase in E-selectin mRNA. We have previously shown that 24h pretreatment of HUVEC with TNF, followed by a 8h TNF washout resulted in high expression of TLR2 mRNA and TLR2 protein at the cell surface. The 8h of TNF washout was shown to be sufficient to bring down to almost basal level the inflammatory response of HUVEC24 (Figure 4A media). TNF pretreatment and 8h TNF washout had no effect on subsequent responses of the HUVEC to TNF or LPS, which shows that the cells were not refractory to subsequent inflammatory stimuli. The higher response to LTA in the TNF-pretreated HUVEC shows the functional relevance of the upregulation of TLR2 (Figure 4A). The TNF pretreatment resulted in an eight to ten-fold higher response of HUVEC to the 19 aPLA and to the β2GP1 immunopurified antibodies (p < 0.0005 and p < 0.05, respectively; two way ANOVA and paired t-test) (Figure 4B) whereas neither the control IgG nor aPLA samples that did not activate monocytes (not shown) had an effect.

We used blocking antibodies to determine the respective roles of TLR2 or TLR4 in the aPLA responses in TNF-pretreated HUVEC. We observed that blocking anti-TLR2 antibodies reduced the responses aPLA between 80 and 95% (p< 0.01; two way ANOVA and paired t-test), whereas anti-TLR4 antibodies were ineffective (Figure 4C).
Analysis of aPLA binding to TLR2 or TLR4

To analyse binding of aPLA to TLR2 or TLR4 we used the Proximity Ligation Assay.27 On monocytes, binding of aPLA to TLR2 was tenfold higher than to TLR4. Binding of aPLA to TLR2 on HEK-TLR2 cells was higher than binding of aPLA to TLR4 on HEK-TLR4 cells. In HUVEC, aPLA bound only to TLR2; the binding signal to TLR4 was comparable to the background control (between 2 and 5 AU). aPLA binding to TLR2 on HUVEC was increased by TNF-pretreatment (Figure 5 and supplementary Figure 4). On all cell types binding of control IgG was much lower than that of aPLA and comparable to the background control, obtained by substituting the primary antibodies with control antibodies.

In addition, binding of one β2GP1-immunopurified IgG preparation to TLR2 on HEK-TLR2 cells and monocytes was 6.2 to 8 ± 1.8-fold (mean ± SE; p<0.01, Mann-whitney test) higher respectively than that of control IgG. Binding of β2GP1-immunopurified IgG to TLR4 on monocytes and on HEK-TLR4 cells was the same as that of control antibodies (p = 1.0).
Discussion

Inflammatory activation of EC, monocytes and platelets appears to be an important factor contributing to the complications of the antiphospholipid syndrome. Under our experimental conditions, a high proportion of APS patients, but none of the healthy controls, had cell-activating antibodies. The present study was undertaken to compare the contribution of TLR2 and TLR4 to inflammatory cell activation by aPLA. We used three cell types to probe the role of these TLRs: monocytes, which express both TLR2 and TLR4, EC, which constitutively express TLR4 and in which TLR2, but not TLR4 is upregulated by TNF, and a model system of HEK293 cells, genetically modified to stably express either TLR2 or TLR4. In this study, we observed that the same aPLA preparations were able to activate monocytes, EC and HEK293 expressing TLR2. Noticeably, aPLA displayed their activating activity by interacting with TLR2, but not with TLR4. Our conclusions are based upon four distinct results: a) monocyte and HUVEC activation by aPLA was inhibited by antibodies to TLR2, but not by antibodies to TLR4; b) pretreatment of HUVEC with TNF increased TLR2 expression, but not TLR4 expression, and resulted in an increased subsequent inflammatory response to aPLA; c) HEK293 TLR2 cells are activated by aPLA and LTA, whereas HEK293 cells stably expressing TLR4/MD2 do not respond; d) in the three different cell types we observed an interaction between aPLA and TLR2, whereas little interaction was seen between aPLA and TLR4, and none between control IgG and TLR2 or TLR4.

The role for TLR2 in cell activation by aPLA apparently contrasts with the pathogenic role for TLR4 described in a mouse thrombosis model. Conceivably, under conditions of chronic or acute inflammation, TLR4-dependent induction of TLR2 expression by EC might provide a mechanism by which an absence of functional TLR4 reduces the prothrombotic effect of aPLA. This notion is in agreement with the two hit hypothesis for thrombotic complications of APS. Release of gut flora-derived endotoxin into the blood circulation might result in a low level of chronic inflammation, that is sufficient to increase TLR2 expression on EC in wild type mice, but not in TLR4-deficient mice. To clarify this issue it will be important to determine in vivo TLR2 expression in large vessels in mouse raised under sterile conditions, under normal conditions or after challenge with inflammatory stimuli. In addition, the thrombogenic effects of aPLA should be studied in parallel in wild type mice, TLR2-deficient mice and TLR4-deficient mice.

The requirement for TLR2 in aPLA-mediated activation of HEK-293 cells, of HUVEC and of monocytes, raises the question as to an interaction between aPLA and TLR2. On all three
cell types tested, we observed binding of aPLA and β2GP1-immunopurified IgG to TLR2 and an increased binding to TLR2 on TNF-pretreated than non-stimulated HUVEC. We observed some binding of aPLA to TLR4, but binding to TLR2 was between 6 to 10 times higher. Our results are in agreement with the binding of β2GP1 only to TLR2, as determined by mass spectrometry analysis after crosslinking of plasma membrane proteins of Eahy926 human endothelial-like cells. In contrast, one report observed co-immunoprecipitation of β2GP1 and TLR4 but did not study co-immunoprecipitation with TLR2. The localization of TLR2 and TLR4 in the same lipid rafts may explain why the different binding studies found some interactions between aPLA or β2GP1, with TLR4.

In this study around half of the aPLA samples were able to activate monocytes and EC. Previously, we observed that only a subset of aPLA samples were able to activate EC. In other studies using freshly isolated monocytes, measurements of tissue factor mRNA revealed that only 66% of APS patients had activated circulating monocytes. As the aPLA concentrations used for cell activation (500 µg/ml) were 20-fold lower than IgG concentrations in vivo, we cannot exclude that some of the negative aPLA samples contained levels of activating antibodies that were under the amount necessary to activate monocytes or EC in vitro tests. Our results do not rule out the alternative hypothesis that aPLA preparations that did not activate monocytes or endothelial cells might increase the risk for adverse clinical outcomes through other mechanisms, such as inhibition of the protein anticoagulant pathway or pre-activation of platelets. The design of the study, as well as the limited number of aPLA samples used, does not allow to draw conclusions as to an association between cell activating antibodies and clinical profile or pattern of positivity in the routine clinical aPLA assays. To resolve this very important clinical issue it will be necessary to undertake specifically designed studies on a much larger scale, using well-defined clinical criteria and standardized aPLA assays.

We observed that TLR2 blockade consistently reduced cell activation responses to aPLA by 50% on average, whereas responses to LTA were reduced by over 80%. Several explanations may be proposed to explain this apparent discrepancy: a) the site on TLR2 that interact directly or indirectly with aPLA may differ from the interaction site of LTA; b) the concentration of the blocking antibodies was chosen to be optimal for inhibition of LTA responses and may not be sufficient for inhibition of aPLA, which are added in amounts exceeding those of the blocking antibodies; c) the affinities of the aPLA toward their cell-activating targets may differ between patients; d) It cannot be ruled out that for some patients other receptors contribute. Our present results do not allow to discriminate between these possibilities.
A clinically important target of aPLA is \( \beta_2 \)GP1. In agreement with a pathogenic role for anti-\( \beta_2 \)GP1 antibodies, we observed activation of all three tested cell types with \( \beta_2 \)GP1-immunopurified antibodies. This suggests that \( \beta_2 \)GP1 can mediate cell activation. We did not observe any association between the cell activation potential of aPLA and their titer in routine anti-\( \beta_2 \)GP1 or aCL ELISAs neither in LA assays. A similar discrepancy between aPLA titers in routine assays and activation potential has been observed previously in a study using U937 monocyte-like cells.\(^{35}\) In contrast, titers of aCL and anti-\( \beta_2 \)GP1 antibodies correlated with the degree of expression of TF by monocytes freshly isolated from APS patients.\(^{34}\) It remains to be established to what extent these discrepancies are due to inadequacies of routine aPLA assays,\(^{24,75,36}\) to the recognition by patient-derived aPLA of different \( \beta_2 \)GP1 epitopes\(^ {37}\) or \( \beta_2 \)GP1 conformations,\(^ {38}\) of which only some may have cell activating potential. Also, we cannot exclude that other target proteins are capable of mediating cell activation. Specifically designed studies involving much greater numbers of patients than used thusfar are required to clarify these issues.

A hallmark of TLR family proteins is their cooperation with cofactor proteins to form a multivalent “sensing apparatus.”\(^{15,16,39,40}\) One of these, CD14, is not essential for cell activation, but enhances cell activation via TLR2 and TLR4. Blocking antibodies to CD14 reduced the inflammatory activation of monocytes by aPLA and LTA and in HEK293 cells expressing both TLR2 and CD14 the reaction to aPLA and LTA was stronger than in HEK293 expressing TLR2 alone. Our results imply a role for CD14 in increasing the sensitivity of monocytes for activation by aPLA. In EC, CD14 is unlikely to play such a role, because CD14 is only weakly expressed on HUVEC and down regulated after inflammatory stimulation.\(^{24}\) Furthermore, besides CD14, other TLR2 co-receptors such as CD36,\(^ {46}\) CD11a (integrin \( \alpha_L \))\(^ {47}\) and CD61 (integrin \( \beta_3 \))\(^ {48}\) are known to modulate TLR2 function. It may be of interest to evaluate a possible contribution of these co-receptors for cell activation by aPLA.

Several receptors have been involved in cellular and platelet responses to aPLA, including annexin 2,\(^ {41-43}\) ApoER2,\(^ {44}\) and GPIb\( \alpha \).\(^ {45}\) Our study shows that TLR2 is another one. It remains to define whether annexin 2, ApoER2 and GPIb\( \alpha \) might form heterocomplexes with TLR2 or whether subgroups of antiphospholipid antibodies deal preferentially with one or other receptor. The fact that different receptors are found involved in cell activation by aPLA may reflect the heterogeneity of these antibodies.

Previous studies have shown that aPLA activate both monocytes and endothelial cells\(^ {33,49}\) and that leukocyte adhesion molecules on EC are important for the thrombogenic effects of aPLA.\(^ {29,50}\) The increased expression of TF in monocytes treated with aPLA implies a role for
these autoantibodies in TF induction.\textsuperscript{3,51,52} The aPLA-induced activation of resting EC, suggests that the low amounts of TLR2 expressed by these cells are sufficient for generating a detectable inflammatory response. However, under conditions of increased TLR2 expression, such as that observed after TNF-pretreatment, a much higher degree of EC activation is obtained. From these results, we may infer a model by which aPLA increase the risk of thrombotic complications. This model has the following aspects: a) In APS patients both monocytes and EC are weakly activated; b) an exogenous inflammatory stimulus, such as LPS or TNF, leads to an increased TLR2-dependent activation of the endothelium and enhanced interaction between monocytes and the endothelium; c) an additional thrombogenic stimulus, such as stasis, may then be sufficient to promote the formation of an intravascular thrombus.

In conclusion, our results provide evidence for a role of TLR2 in the activation of EC and monocytes by aPLA from a large proportion of APS patients. Further progress in the understanding of APS requires the parallel analysis of all proposed pathogenic mechanisms, as well as the conduction of standardized routine aPLA assays, for a large number of patient samples, to determine whether there is common underlying pathogenic mechanism for all aPLA or whether the APS is composed of several distinct syndromes. Our observation that TLR2 expression by EC, as well as responses of EC to aPLA, increased after an inflammatory stimulus may offer an explanation for the clinical observation that aPLA normally circulate without thrombotic effects and why complications of the antiphospholipid antibody syndrome frequently occur in association with bacterial or viral infections.
Acknowledgments

We would like to extend special thanks to Dr Bernadette Mermillod for her assistance in performing biostatistical analysis.

Authorship and Conflict of Interest Statements

Contribution of the authors to various aspects of the manuscript: designed the research: NS, EK; performed research: NS, CF; provided samples: FB, SDG, DB; analyzed data: NS, EK, GR, PdM; wrote the paper: NS, EK, GR, PdM.

The authors declare no conflicts of interest.
References


Table 1: **Clinical data and cell activation potential of aPLA**

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NB. There was no significant association between cell activation potential and patient characteristics or positivity in a particular aPLA assay.
Legends

Figure 1: Monocyte response to aPLA in the presence of anti-TLR2, anti-TLR4 or anti-CD14 antibodies.
A) Monocytes were incubated for 4 h with 32 aPLA or 19 control IgG (CTL) at 500 µg/ml. Cell activation was assessed by quantification of TNF in cell supernatants by ELISA. The cut off value (grey line) for monocytes activation was set to 0.44 ng/ml (mean of control IgG + 3 SD).
B and C) Blocking antibodies to TLR2, TLR4 or CD14, as well as the corresponding isotype matched control antibodies (Control) (10 µg/ml) were incubated with monocytes for 30 min followed by a 4h incubation with the 19 activating aPLA or control IgG (CTL) at 500 µg/ml, or two aPLA preparations immunopurified on β2GP1 (100 µg/ml). (B) TNF secretion was quantified by ELISA in cell supernatants. (C) Changes in tissue factor (TF) mRNA levels were evaluated by qPCR. (D) Tissue Factor activity was quantified as described in M&M and expressed as pM of generated FXa. Statistical analysis indicated a significant difference in monocyte stimulation by aPLA in presence versus in absence of anti-TLR2 or anti-CD14 antibodies (p< 0.001; two-way ANOVA and paired t-test).

Figure 2: TLR2- or TLR4-dependent HEK activation by aPLA and control IgG (CTL).
HEK-Blue-2 or HEK-Blue-4 were incubated with 500 µg/ml of aPLA from 32 patients or 19 Control IgG for 16h. Cell activation was quantified by measuring change in OD at 650 nm, which corresponds to sAP activity. The activation of HEK-Blue-2 and HEK-Blue-4 by aPLA is significantly different (p< 0.0001 two-way ANOVA and paired t-test).

Figure 3: Role of CD14 in TLR2 activation by LTA or aPLA.
A. HEK-Blue-2 (CD14) and HEK-TLR2 (no CD14) were incubated with 500 µg/ml of the 19 aPLA or control IgG or two aPLA preparations immunopurified on β2GP1 (100 µg/ml) for 16h. Then, supernatants were collected and IL-8 quantified by ELISA. B and C. Dose response experiments for LTA (B) or aPLA from 3 patients (C) were made under the same experimental conditions. Data are expressed as means ± SE, n=5.
Cell number was verified at the end of the assay using a cell viability assay (see materials and methods) and was found to be very similar for both cell lineages (OD_{490nm} HEK-Blue-2/OD_{490nm} HEK-TLR2: 1.15 ± 0.03).
Figure 4: E-Selectin expression in HUVEC stimulated by aPLA.

HUVEC were pre-incubated for 24h with medium (HUVEC) or with 100 ng/ml TNF (TNF-pretreated HUVEC), followed by an 8h washout with regular medium.

A. Cells were further incubated for 4h with 100 ng/ml TNF, 1 µg/ml LPS, 10 µg/ml LTA or medium alone. B. Cells were further incubated for 4h with 500 µg/ml of the aPLA (n=19), 100 µg/ml of β2GP1-immunopurified IgG (n=2) or 500 µg/ml of control human IgG. Changes in E-Selectin mRNA level were evaluated by qPCR. Unstimulated HUVEC were taken as reference for basal E-Selectin expression. Note that the cellular responses to 100 ng/ml TNF or 1 µg/ml LPS were similar in the TNF-pretreated cells and in the control cells, which shows that the cells had not become refractory to a subsequent inflammatory stimulus. The E-Selectin mRNA level observed for TNF-pretreated HUVEC media condition corresponded to the remaining effect of the 24h TNF stimulation followed by the 8h washout. Data are derived from 4 different cell preparations and are expressed as means ± SE.

C. Cells were incubated for 30 min with TLR2- or TLR4-blocking antibodies followed by 4h incubation with 500 µg/ml of six aPLA preparations selected among the highest stimulators, 1 µg/ml LPS or 10 µg/ml LTA for 4h. Changes in E-Selectin mRNA levels were evaluated by qPCR. The results are expressed as the ratio of the E-selectin response of TNF pretreated HUVEC incubated with the blocking anti-TLR2 antibodies versus the E-selectin response of TNF-pretreated HUVEC. Data are derived from five different HUVEC preparations and expressed as means ± SE, n =5.

Figure 5: Binding of aPLA to TLR2 on HUVEC, HEK-293 and monocytes.

Binding of aPLA to TLR2 or TLR4 on (A) monocytes, (B) HEK-TLR2 cells and HEK-TLR4 cells; and (C) HUVEC and TNF-pretreated HUVEC (TNF) was quantified by the Proximity Ligation Assay. Cells were incubated for 1h with 100 µg/ml of one aPLA or control IgG (CTL) and then for 1h with 10 µg/ml of polyclonal rabbit anti-TLR2 or -TLR4 antibodies. Thereafter, cells were incubated with probe-labelled secondary antibodies and development of fluorescent signal as described in the methods section. Binding was evaluated by quantifying the fluorescence area per cell and was expressed in arbitrary units, proportional to the experimental condition that gave the highest fluorescence signal for each cell type. The average number of counted cells per image was 60±2 for HEK-TLR2, 59±3 for HEK-TLR4, 40±10 for HUVEC, 42±7 for TNF-treated HUVEC and 21±6 for monocytes.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

Monocytes

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HUVEC

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Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies

Nathalie Satta, Egbert K.O. Kruithof, Céline Fickentscher, Sylvie Dunoyer-Geindre, Françoise Boehlen, Guido Reber, Danielle Burger and Philippe de Moerloose