Anti-β₂-glycoprotein I antibody with DNA binding activity enters living monocytes via cell surface DNA and induces tissue factor expression

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Summary

Autoantibodies characteristic for anti-phospholipid syndrome (APS) and systemic lupus erythematosus (SLE) are anti-β₂-glycoprotein I (β₂GPI) antibodies and anti-DNA antibodies, respectively, and almost half of APS cases occur in SLE. Anti-β₂GPI antibodies are recognized to play a pivotal role in inducing a prothrombotic state, but the precise mechanism has not been fully elucidated. In a widely accepted view, binding of anti-β₂GPI antibodies to cell surface β₂GPI in monocytes and endothelial cells triggers the Toll-like receptor 4-myeloid differentiation primary response 88 (TLR)-4-MyD88 signaling pathway which leads to activation of p38 mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase kinase1/extracellular signal-regulated kinases (MEK-1/ERK) and/or nuclear factor kappa B (NF-κB) and expression of tissue factor (TF). However, resting cells do not express substantial amounts of TLR-4. Previously, we generated a mouse monoclonal anti-β₂GPI antibody WB-6 and showed that it induced a prothrombotic state – including TF expression on circulating monocytes – in normal mice. In the current study, we aimed to clarify the mechanism of interaction between WB-6 and resting monocytes, and found that WB-6 exhibits binding activity to DNA and enters living monocytes or a monocytic cell line and, to a lesser extent, vascular endothelial cells. Treatment of the cells with DNase I reduced the internalization, suggesting the involvement of cell surface DNA in this phenomenon. Monocytes harboring internalized WB-6 expressed TF and tumor necrosis factor (TNF)-α which, in turn, stimulated endothelial cells to express intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). These results suggest the possibility that a subset of anti-β₂GPI antibodies with dual reactivity to DNA possesses ability to stimulate DNA sensors in the cytoplasm, in addition to the cell surface receptor-mediated pathways, leading to produce proinflammatory and prothrombotic states.

Keywords: anti-DNA antibodies, anti-phospholipid antibodies, anti-phospholipid syndrome, endocytosis, systemic lupus erythematosus

Introduction

Anti-phospholipid syndrome (APS) is an autoimmune disorder defined by the presence of clinical features such as arterial and venous thrombosis, or pregnancy morbidity, associated with anti-phospholipid antibodies. These are usually tested for in clinical laboratories by assessing antibodies to cardiolipin (CL), β₂-glycoprotein I (β₂GPI) and the activity of lupus anti-coagulant (LA) [1,2]. Approximately half of patients do not have underlying diseases and are called primary APS, while others are called secondary APS and suffer from other diseases, mostly systemic lupus erythematosus (SLE) [3]. The reason why APS tends to occur frequently in SLE has not been clearly explained.

Extensive studies have revealed that pathologically relevant anti-phospholipid antibodies are not those that bind directly to phospholipids, but those that bind indirectly
to phospholipids through phospholipid binding proteins, such as β2GPI and prothrombin. In particular, the presence of anti-β2GPI antibodies with LA activity has been shown to correlate closely with thrombotic events in APS patients [4]. In a previous study, we generated a monoclonal antibody WB-6 from an (NZW × BXSB) F1 male mouse [5]. This antibody binds to CL-β2GPI and possesses LA-like activity. On in-vivo administration, WB-6 induced a prothrombotic state in normal mice, including tissue factor (TF) expression by circulating monocytes, which could be prevented by treatment with a nuclear factor kappa B (NF-kB) inhibitor. Thereafter, we were interested to explore interactions between WB-6 and relevant cells.

To activate prothrombotic mechanisms, it would be expected that anti-β2GPI antibodies need to bind to cell surface β2GPI, which is a plasma protein of approximately 50 kDa and consists of five sushi-domains. It exists in two conformations: a closed circular conformation in plasma and an open fishhook-like shape when the C-terminal domain V binds to negatively charged cell surface receptors [6]. Major pathological anti-β2GPI antibodies do not bind to the β2GPI in the former structure, but recognize the cryptic epitope on the N-terminal domain I exposed in the latter, surface-bound form [7]. Of the several candidate receptors for β2GPI the best known is phosphatidylserine, which is normally located in the inner leaflet of the cell membrane. Phosphatidylserine is exposed on the surface of apoptotic cells, but can also be externalized by stimulation with proinflammatory cytokines followed by activation of phospholipid scramblase 1 [8]. Other proposed receptors for β2GPI on monocytes or endothelial cells include annexin A2, but this lacks a cytoplasmic tail and requires a co-receptor to activate the intracellular signaling pathways [9,10]. Toll-like receptor (TLR)-4 is the best-characterized co-receptor in this respect [11–13], but it may not be expressed on resting cells at levels high enough to facilitate activation by anti-β2GPI antibodies [14,15]. The present study was therefore undertaken to investigate how WB-6 contacts and activates resting monocytes, resulting in their TF expression.

**Materials and methods**

**Cells and monoclonal antibodies**

The study protocol was approved by TMDU Faculty of Medicine Ethics Committee (M2000-1480). Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by density gradient centrifugation over Ficoll-Conray solution. PBMCs and human monocytic leukemia cell line THP-1 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 mM non-essential amino acids. Human umbilical vein endothelial cells (HUVECs) were purchased from Takara Bio (Kusatsu, Shiga, Japan), cultured in PromoCell Growth Medium (Takara Bio), and used at passage 4 or lower. Monoclonal antibody WB-6 [immunoglobulin (Ig)G2b, κ] was generated in a lupus-prone (NZW × BXSB) F1 mouse [5], and 2C10 (IgG2b, κ) in an MRL/lpr mouse [16]. These monoclonal antibodies were purified from culture supernatants of hybridomas grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 mM non-essential amino acids, by salting-out with half-saturated ammonium sulfate followed by column chromatography with protein G HP Spin Trap (GE Healthcare, Chicago, IL, USA) and dialysis against phosphate-buffered saline (PBS). Final concentrations of lipopolysaccharide (LPS) derived from each antibody preparation in culture media were confirmed to be < 2.3 pg/ml by Limulus Color KY Test (FUJIFILM Wako Chemical, Osaka, Japan).

**Enzyme-linked immunosorbent assay (ELISA)**

Antibody activity to cardiolipin and β2GPI was determined by ELISA, as described previously [5]. For testing DNA-binding activity, ELISA plates (Immulon 2HB; Thermo Scientific, Fremont, CA, USA) were ultraviolet (UV)-irradiated (10 000 µJ/cm² for 2 h) and coated with 5 µg/ml calf-thymus (CT) DNA (Sigma Aldrich, St Louis, MO, USA) in Tris-buffered saline (25 mM Tris, 140 mM NaCl, pH 7.4; TBS) overnight at 4°C. After washing with TBS, blocking with 1% bovine serum albumin (BSA)-TBS and washing with TBS, monoclonal antibodies diluted in 1% BSA-TBS were incubated in the plates for 1 h at room temperature. After washing with TBS, bound antibodies were detected using alkaline phosphatase-labeled antimouse IgG antibody and p-nitrophenyl phosphate. In a competitive assay, monoclonal antibodies were preincubated in microtubes with the inhibitors CT-DNA, poly (dA-dT) or poly (dT) in 1% BSA-TBS for 1 h, and then put into the CT-DNA-coated ELISA plates.

**Fluorescence immunocytochemistry**

THP-1 cells, HUVECs or PBMCs were seeded into 48-well culture plates or Lab-Tek Chamber Slides (Thermo Fisher) and grown until 90% confluent. After removing the supernatant, 2C10, WB-6 or isotype-matched control IgG (R&D Systems, Minneapolis, MN, USA) containing fresh medium was added to the wells, and incubated for 2 h at 37°C in a CO₂ incubator. Unbound antibody was then removed by washing with ice-cold PBS, and the cells were fixed with 4% paraform-aldehyde-PBS for 10 min, followed by neutralization with 100 mM glycerine-PBS. After permeabilization with 0.1% Triton X-PBS and blocking with 1% goat serum-PBS for 30 min, the cells were washed with...
PBS and stained with Alexa Fluor 488-labeled goat antimouse IgG (Abcam, Cambridge, MA, USA) for 1 h at room temperature. In some experiments, after incubation with 2C10, WB-6 or isotype-matched IgG cells were washed twice with PBS and then stained with Alexa Fluor 488-labeled anti-IgG directly before fixation and permeabilization. The cells were observed using a fluorescence microscope (Keyence, Osaka, Japan) or analyzed by flow cytometry (CytoFLEX, Beckman Coulter). Some pictures were taken using a confocal laser scanning microscope TCS SP8 (Leica, Tokyo, Japan). In some experiments, the cells were treated with 200 Kunitz µ/ml bovine pancreas DNase I (Sigma Aldrich) for 1 h in medium containing 5 mM MgCl₂ before incubation with monolarclonal antibodies. In some experiments, cell surface expression of phosphatidylserine was tested after 2 h incubation of THP-1 cells with WB-6 using Annexin V-Biotin Apoptosis Detection Kits (BioVision) and fluorescein isothiocyanate (FITC)-streptavidin.

Detection of TF mRNA, TF protein and TF activity
PBMCs were incubated with 10–25 µg/ml WB-6, isotype-matched IgG or 25–100 ng/ml LPS (as a positive control) for 3 h to detect TF mRNA or for 5 h to detect TF protein. To detect TF mRNA, total RNA was extracted from PBMCs using Isogen II (Nipon Gene, Tokyo, Japan) and cDNA was generated using SuperScript III First-Strand Synthesis System (Thermo Fisher). Real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed using the Illumina Eco System (Illumina, San Diego, CA, USA) with primers specific to human TF (forward: 5′-CAGGGAATGTGGAGACACC-3′, reverse: 5′-ATTGTTGGCTGTCCGAGGTT-3′), and normalized against the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

To detect TF at protein level, cells were stained with phycoerythrin (PE)-conjugated anti-human CD142 antibody (Thermo Fisher) for 20 min, and analyzed by flow cytometry. TF activity was tested using assaySense Human Tissue Factor Chromogenic Activity Kit (Assaypro, St Charles, MO, USA). In this assay, cell lysates were incubated with Factor VII and Factor X for 30 min at 37°C, followed by further incubation with chromogenic Factor Xa substrate, according to the manufacturer’s protocol.

Detection of adhesion molecules on HUVECs
THP-1 cells were stimulated with or without 25 µg/ml WB-6 or isotype-matched IgG for 4 h in 48-well culture plates, after which the supernatants (conditioned media) were collected by centrifugation, and kept at –80°C until use. A panel of inflammatory cytokines and chemokines in the conditioned media was measured by Multi-Analyte Flow Assay Kit (BioLegend, San Diego, CA, USA). HUVECs were cultured in 48-well culture plates and grown until 90% confluent, then washed with medium and stimulated directly with 25 µg/ml WB-6 or isotype-matched IgG, or indirectly with the above-described conditioned media for 6 h. In some experiments, HUVECs were stimulated in the presence of 1 µg/ml anti-tumor necrosis factor (TNF)-α neutralizing monoclonal antibody (AB_468487; Thermo Fisher). After washing with cold PBS, fixation with 0.5% paraformaldehyde PBS and washing with PBS, cells were incubated with FITC-labeled mouse anti-human CD54 intercellular adhesion molecule 1 (ICAM-1) IgG (Thermo Fisher) or PE-labelled anti-human CD106 [vascular adhesion molecule 1 (VCAM-1)] IgG (Thermo Fisher) for 30 min. Then, after washing with PBS, cells were detached by treatment with 0.05% porcine trypsin with 0.02% ethylenediamine tetraacetic acid (EDTA) (Sigma Aldrich) for 1–2 min and provided with flow cytometry. TF activity in the lysates of these stimulated HUVECs was also tested using a TF chromogenic activity kit, described above.

Statistical analysis
Data were analyzed by a two-tailed unpaired parametric t-test using Prism version 5.0c for Mac OS X (GraphPad Software, La Jolla, CA, USA).

Results
Monoclonal antibody WB-6 binds to DNA as well as to CL-β₂GPI
We have previously reported that WB-6 preferentially binds to CL-β₂GPI complexes compared to CL alone or β₂GPI alone [5]. To further investigate the specificity of WB-6, we first confirmed the previous findings using ELISA (Fig. 1a). However, we also noticed unanticipated binding of WB-6 to calf-thymus (CT) native DNA, as shown in Fig. 1b. We then compared WB-6 with 2C10, which is an authentic anti-double-stranded (ds) DNA antibody that does not bind to single-stranded (ss) DNA [16] or CL-β₂GPI. WB-6 bound to CT-DNA in a dose-dependent manner with slightly less affinity than 2C10. In an inhibition ELISA, WB-6 showed much higher affinity for the synthetic ssDNA, poly(dT) than to synthetic dsDNA or CT-DNA, suggesting that the binding site for WB-6 includes the sugar-phosphate backbone of DNA (Fig. 1c).

WB-6 enters living THP-1 and HUVEC cells
Because we had been interested in sporadically reported topics on internalization of anti-DNA antibodies into living cells, and now found that WB-6 possesses DNA-binding properties, we tested whether WB-6 enters live
cells. THP-1 cells were cultured with WB-6, and after washing, fixation and permeabilization we sought to detect it inside the cells using Alexa Fluor 488-labeled goat anti-mouse IgG. We found that WB-6, but not the isotype-matched control IgG, entered the cytoplasm of THP-1 cells within 2 h at 37°C (Fig. 2a). Confocal images revealed many small specks mainly formed in the cytoplasm, close to the cell surface, suggesting that they are in the endosomes. The dsDNA-specific antibody 2C10 also entered the cytoplasm of THP-1 even more robustly and often it appeared to enter the nuclei. Confocal images showed many small specks, mainly in the perinuclear region and possibly in the nucleoplasm. In a separate experiment, we confirmed that 2C10 entered the nuclei by electron microscopy (data not shown).

After 2 h incubation with WB-6, the cells were not stained by annexin V, indicating that they were not apoptotic at this time-point (Fig. 2b). When we added Alexa Fluor 488-labeled goat anti-mouse IgG before fixation and permeabilization, virtually all cells remained unstained, as shown in Fig. 2c. These results again demonstrate the integrity of the cell membrane, because the second antibody could not enter the cells. In addition, these findings suggest that most of WB-6 and 2C10 did not remain on the cell surface after 2 h, or some cell surface antibodies, if they remained, could not be detected because they were not accumulated like the antibodies in endosomes.

Antibody internalization was also tested using HUVECs. 2C10, but not the isotype-matched control IgG, entered the cells, similar to the results with THP-1 cells, while WB-6 entered the cells with apparently less efficiency (Fig. 2d).

Cell surface DNA is involved in the internalization of WB-6

It is known that DNA or nucleosomes can be found attached to the cell surface both in vitro and in vivo. We therefore hypothesized that the cell surface DNA is involved in the internalization of WB-6. To test this, THP-1 cells were treated with DNase I before incubation with WB-6. This resulted in decreased internalization of WB-6 into THP-1 cells (Fig 3a). Quantitative analysis by flow cytometry showed that treatment of THP-1 cells with DNase I significantly decreased both the percentage of fluorescence-positive cells and the mean fluorescence intensity (Fig. 3b,c). Similarly, the internalization of WB-6 into HUVECs was also suppressed by treatment with DNase I (data not shown). These results suggest that WB-6 enters the cells at least in part, through binding to the cell surface DNA.

WB-6 enters normal monocytes and induces TF expression

To test whether WB-6 also enters normal resting cells, the antibody was added to cultures of PBMCs obtained from healthy volunteers. Internalization of WB-6 was observed by fluorescence microscopy after 2 h incubation in only a small fraction of the PBMCs with slightly larger cell size than other cells (Fig. 4a). Using flow cytometry, PBMCs were separated into two major populations representing monocytes and lymphocytes designated P1 and P2, respectively in Fig. 4b. Nearly 90% of the cells in P1 were positive for the monocyte marker CD14 (Fig. 4c). When PBMCs were incubated with WB-6 fluorescence-positive cells were detected selectively in the P1 population, indicating that WB-6 enters normal monocytes but not lymphocytes (Fig. 4d–g).

To test the biological effect of WB-6 internalization into monocytes, PBMCs were incubated with WB-6 and the expression of TF was tested by qRT–PCR and flow cytometry. After 3 h incubation WB-6, but not the isotype-matched control IgG, induced TF mRNA expression in PBMCs (Fig. 5a). After 5 h incubation, TF expression at protein level was observed in approximately 33% of the
Dual-reactive antibody to β2GPI and DNA

(a) | Medium | IC IgG | WB-6 | 2C10
---|---|---|---|---
Nuclei
2nd Ab
Merge
Confocal image

(b) | Medium | WB-6 | Staurosporine (6h)
---|---|---|---
Annexin V
Merge

(c) | WB-6 | WB-6
---|---|---
2nd Ab after permeabilization | 2nd Abs before permeabilization

(d) | Medium | IC IgG | WB-6 | 2C10
---|---|---|---|---
Nuclei
IgG
Merge
Confocal image
monocyte-rich population (Fig. 5b). This TF was confirmed to have procoagulant activity using a chromogenic assay kit, as described in Materials and methods (Fig. 5c).

**WB-6 activates HUVECs indirectly through activation of THP-1**

It has been reported that anti-phospholipid antibodies in APS patients induce expression of adhesion molecules as well as TF on vascular endothelial cells, and that this results in enhancement of thrombus formation [19,20]. To test whether WB-6 is able to activate HUVECs directly to induce adhesion molecules or TF, HUVECs were incubated with WB-6. However, no significant expression of VCAM-1, ICAM-1 or TF was observed under our assay conditions (data not shown). In contrast, VCAM-1 and ICAM-1, but not TF, were induced on HUVECs when the cells were exposed to culture supernatant from THP-1 cells that had been incubated with WB-6 (Fig. 6a). These results suggest that HUVECs were activated by cytokines secreted from WB-6-stimulated THP-1 cells. We measured a panel of inflammatory cytokines/chemokines in the conditioned medium and found that it contained high concentrations of TNF-α, monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-8 and a low concentration of IL-23 (Table 1). TNF-α was thought to be the major contributor to the HUVEC activation in our assay, because VCAM-1/ICAM-1 induction was completely inhibited by anti-TNF-α monoclonal neutralizing antibody (Fig. 6b,c).

**Discussion**

Previously, we reported that the monoclonal antibody WB-6 preferentially binds to CL-β2 GPI, and observed that it induced a prothrombotic state in normal mice at least partially as a result of inducing TF expression in circulating monocytes [5]. In the present study, we found that WB-6 possesses dual reactivity to DNA as well as CL-β2 GPI. WB-6 was able to enter living human monocytes, THP-1 cells, and to a lesser extent, HUVECs, via binding to cell surface DNA. While anti-β2 GPI antibodies are known to potentially induce prothrombotic and proinflammatory states in...
monocytes and endothelial cells by binding to cell surface β₂GPI, the present data suggest the possibility that, apart from the TLR-4 axis, signaling pathways originating from cytoplasmic DNA sensors may also be involved in the pathogenic effects of a subset of anti-β₂GPI antibodies.

In 1981, Lafer et al. [21] first demonstrated that some monoclonal antibodies show cross-reactivity between ssDNA and cardiolipin, which provided an explanation of the frequently observed biological false-positive results in serological tests for syphilis in patients with SLE. Pathologically more relevant autoantibodies, however, are those reactive with dsDNA in SLE, and with CL-β₂GPI in APS. From this point of view, the present finding of WB-6 dual reactivity to CL-β₂GPI and dsDNA explains more clearly why a major proportion of secondary APS is associated with SLE. In the literature, we found one example of a monoclonal antibody with a similar specificity to WB-6, designated 3H9 [22]. This antibody binds to phosphatidylserine, but with higher affinity to phosphatidylserine-β₂GPI complex, and also to CT-DNA.

Based on the observation that 3H9 bound to annexin V-positive apoptotic Jurkat cells, but not to annexin V-negative cells, the authors speculated that such antibodies may play a role in the clearance and processing of apoptotic cells. We would agree with that suggestion, but in the current study we documented interactions between WB-6 and living cells and propose another important role for such antibodies in pathogenic processes. With regard to structural requirements for antibody cross-reactivity between proteins and polynucleotides, a computer model has predicted residue–residue interactions across the interface between some anti-DNA antibodies and β₂GPI [23], consistent with the existence of a subset of autoantibodies similar to WB-6 and 3H9.

Studies on internalization of anti-nuclear antibodies into living cells have had a long history since the first report by Alarcon-Segovia et al. in 1978 [24], and the mechanisms responsible for the internalization and the functional consequences thereof seem diverse. Some studies showed amplification effects of cell-surface...
DNA–histone complexes or nucleosomes on anti-DNA antibody internalization [17,25], while another study indicated a role of cell surface myosin-1 for entry [26]. The original study suggested an involvement of Fc receptors in the internalization [24], but anti-DNA antibodies lacking the Fc portion have been reported to also enter living cells [27], and IgG anti-DNA antibodies were also able to enter FcγR-negative HeLa cells [25]. Internalization of anti-DNA antibodies may lead to enhanced apoptosis according to some reports [27,28] but, in contrast, other studies showed that antibody internalization could induce expression of the cell activation markers CD69, CD71 and CD98 on human PBMCs [29] or secretion of TNF-α from a murine macrophage cell line [30]. These diverse effects may have resulted from the different assays employed in each study, but in any event it now seems clear that some populations of anti-DNA antibodies can enter some types of living cells. What we observed in the current study was internalization of WB-6 preferentially into monocytes or a monocytic cell line cells, but not into lymphocytes, through binding to cell surface DNA. Subsequently, the cells were activated to express TF and some proinflammatory cytokines, including TNF-α.

Apart from anti-DNA antibodies, information concerning internalization of anti-phospholipid antibodies are very limited, but we have noted two intriguing studies. The first was reported by Prinz et al. [31], in which they observed that anti-phospholipid antibodies (with no binding activity to β2GPI or DNA) were internalized by monocytc cells and plasmacytoid dendritic cells, and induced translocation of TLR-7 or TLR-8 from the endoplasmic reticulum to the endosome, leading the cells to be sensitized to ligands for TLR-7/TLR-8. The second report was from Viall et al. [32], in which they showed that anti-β2GPI antibodies (DNA binding activity was not mentioned) were internalized by syncytiotrophoblast, and affected the death-regulating function of the mitochondria, causing release of necrotic trophoblast debris relating to pathogenesis of pre-eclampsia. These studies and our

Fig. 5. Induction of tissue factor (TF) expression in monocytes by WB-6. Peripheral blood mononuclear cells (PBMCs) were incubated with WB-6, isotype-matched non-DNA-binding monoclonal antibody kappa monoclonal MPC-11, isotype-matched control immunoglobulin (Ig)G (IC) or lipopolysaccharide (LPS). (a) After 3 h incubation, TF mRNA expression was evaluated by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). (b) After 5 h incubation, the ratio of CD142+ cells in the monocyte fraction was estimated by flow cytometry. (c) After 5 h incubation, amidolytic activity of TF/FVIIa complex in the PBMC lysates was quantitated and expressed as the equivalent standard TF concentrations. Data show a representative of three independent experiments with similar results and the mean ± standard error of the mean (s.e.m.) of triplicate assay. ***P < 0·001.
current observations provide a new focus of interest on the role of anti-phospholipid antibodies in APS pathogenesis.
IL-6 <2·30 <2·30
MCP-1 27·4 424
IL-8 8·80 617
IL-10 <1·51 <1·51
MCP-1 27·4 424
IL-23 3·69 5·33
IL-33 <9·17 <9·17

IL-1β <1·82 <1·82
IL-8 8·80 617
IL-10 <1·51 <1·51
IL-18 <2·22 <2·22
IL-1β <1·82 <1·82

Overall, our findings suggest a scenario in which WB-6 initially comes into contact with cell surface DNA and drives one of the mechanisms of endocytosis. Subsequently, DNA accompanied by the antibody may be recognized by a cytoplasmic DNA sensor such as TLR-9, AIM2 or STING, and ultimately activates the NF-κB pathway to express proinflammatory molecules including TNF-α, MCP-1 and IL-8. In addition, WB-6 induces monocytes to express TF, but it is unlikely that all types of internalized anti-DNA antibodies are able to induce TF, given that only a fraction of SLE patients exhibit APS. In fact, another anti-DNA antibody 2C10, which does not bind to β2 GPI, enters living monocytes and endothelial cells more readily than WB-6 and induces expression of TNF-α [30], but fails to induce TF significantly (our data not shown). The mechanisms responsible for the differential effects of internalized anti-DNA antibodies on cellular function are under investigation. In one widely accepted view, anti-β2 GPI antibodies bind β2 GPI on the surface of monocytes or endothelial cells and activate the TLR-4-myeloid differentiation primary response 88 (MyD88) signaling pathway leading to activation of p38 mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase kinase1/extracellular signal-regulated kinases (MEK-1/ERK) and/or NF-κB, and expression of TF [9,13]. However, resting cells do not exhibit many anionic phospholipids or other receptors for β2 GPI, nor the co-receptor TLR-4, and therefore β2 GPI may not bind to the cell surface at a sufficiently high density to cause pathological reactions [14,15]. A possible solution to this problem is a two-hit hypothesis, in which inflammatory stimuli are necessary for the occurrence of thrombotic events in addition to the anti-β2 GPI-induced thrombophilic state [9]. In the case of WB-6, it enters resting monocytes and supposedly activates the innate immunity system to produce proinflammatory cytokines, which may result in phospholipid scramblase 1 up-regulation [8] and phosphatidylserine exposure, up-regulation of TLR-4 and facilitates β2 GPI binding to the surface of living cells. Furthermore, we observed that proinflammatory cytokines produced following WB-6 internalization activated endothelial cells to express ICAM-1 and VCAM-1, which is recognized as another mediator of thrombus formation [19]. Activation of HUVECs may be ascribed mainly to TNF-α, but other factors secreted from WB-6-stimulated THP-1 cells may also play a role in thrombogenesis by other mechanisms; for example, MCP-1 is known to induce production from human monocytes in the presence of anti-dsDNA antibody-positive IgG from SLE patients by activating the TLR-9 pathway and NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome [36].
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TF expression on THP-1 cells [37]. Besides, we observed previously that TNF-α induced expression of chemokines CX3CL1 and CCL5 by HUVECs, and these increased adhesive activity and aggregation activity of platelets, respectively [38]. Thus, a subpopulation of anti-β2GPI antibodies has the potential to induce a prothrombotic state through unique mechanisms. WB-6 did not directly induce TF expression in HUVECs, but whether this resulted from less internalization of WB-6 into HUVECs than THP-1 remains to be studied.

In summary, we have described for the first time, to our knowledge, the pathogenic activity of a dual-reactive monoclonal antibody that binds to CL-β2GPI and to DNA. It entered resting monocytes or a monocytic cell line through binding to the cell surface DNA and induced cell surface expression of TF and secretion of inflammatory cytokines, including TNF-α which, in turn, stimulated vascular endothelial cells to express ICAM-1 and VCAM-1. Although intracellular signaling pathways responsible for these events remain to be identified, those linked to any cytoplasmic DNA sensors are supposed to be involved in addition to the widely accepted TLR-4-Md88 pathway. A subset of anti-phospholipid antibodies with these characteristics, whether they are monoclonal antibodies with dual-reactivity or polyclonal mixture of anti-β2GPI and anti-DNA in patient plasma, may play a prominent role in the pathogenesis of secondary APS associated with SLE.

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Disclosure

All the authors have no conflicts of interest to disclose.

Author contributions

S. V. performed the experiments and wrote the paper; M. S., Y. W., K. I. and O. H. performed the experiments; T. K. designed the study and revised the paper.

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