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Anti-β_2-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-β_2-glycoprotein I (β_2GPI) antibodies and anti-DNA antibodies, respectively, and almost half of APS cases occur in SLE. Anti-β_2GPI 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-β_2GPI antibodies to cell surface β_2GPI 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-β_2GPI 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-I) and vascular cell adhesion molecule 1 (VCAM-I). These results suggest the possibility that a subset of anti-β_2GPI 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), β_2-glycoprotein I (β_2GPI) 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

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to phospholipids through phospholipid binding proteins, such as β_2GPI and prothrombin. In particular, the presence of anti-β_2GPI 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-β_2GPI 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-κB) inhibitor. Thereafter, we were interested to explore interactions between WB-6 and relevant cells.

To activate prothrombotic mechanisms, it would be expected that anti-β_2GPI antibodies need to bind to cell surface β_2GPI, 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-β_2GPI antibodies do not bind to the β_2GPI 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 β_2GPI 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 β_2GPI 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-β_2GPI 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 β_2GPI 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/cm^2 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 anti-mouse 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 CO_2 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 glycine-PBS. After permeabilization with 0·1% Triton X-PBS and blocking with 1% goat serum-PBS for 30 min, the cells were washed with

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PBS and stained with Alexa Fluor 488-labeled goat anti-mouse 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 $MgCl_2$ before incubation with monoclonal 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 (Nippon 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′-CAGGGAATGTGGAGAGCACC-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℃, 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℃ 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-I) 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-β_2GPI

We have previously reported that WB-6 preferentially binds to CL-β_2GPI complexes compared to CL alone or β_2GPI 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-β_2GPI. 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

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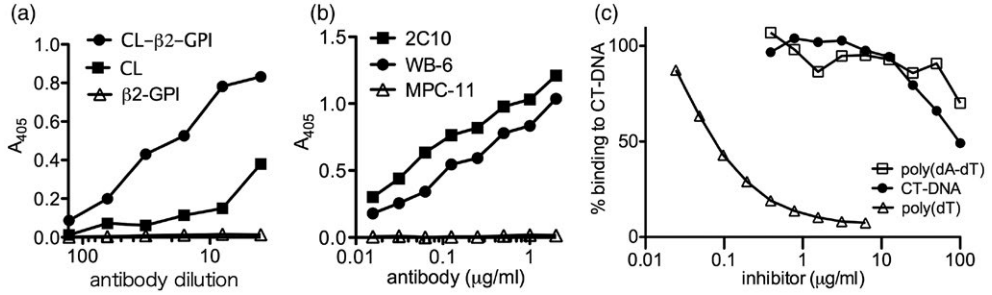

Fig. 1. Dual-reactivity of WB-6 to cardiolipin-β_2-glycoprotein I (CL-β_2GPI) and DNA. (a) Direct-binding enzyme-linked immunosorbent assay (ELISA) to test the binding activity of WB-6 to β_2GPI, CL and CL-β_2GPI. (b) Direct-binding ELISA to show the binding activity of WB-6 and 2C10 to calf-thymus (CT) native DNA. Mouse immunoglobulin (Ig)G2b, kappa monoclonal MPC-11 is an isotype-matched control without DNA-binding activity. (c) Inhibition ELISA in which binding activity of WB-6 to calf thymus (CT)-DNA was inhibited by preincubation with poly(dT), poly(dA-dT) or CT-DNA.

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℃ (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* [17,18]. 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

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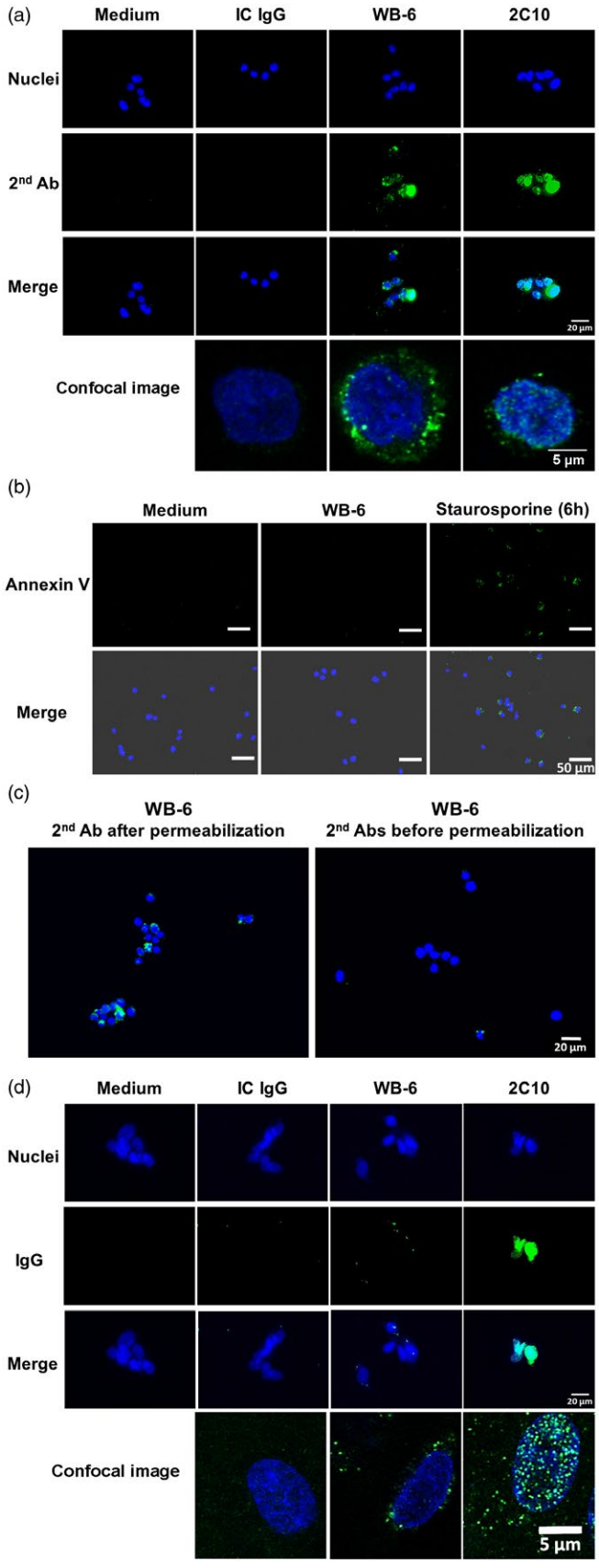
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(d)
Medium
IC IgG
WB-6
2C10
Nuclei
IgG
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Confocal image
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Fig. 2. Internalization of WB-6 into living human monocytic leukemia cell line THP-1 cells and human umbilical vein endothelial cells (HUVECs). (a) THP-1 cells were incubated with 25 μg/ml WB-6, isotype-matched control (IC) or 5 μg/ml 2C10 for 2 h. After washing, fixation, permeabilization and blocking, internalized immunoglobulin (Ig)G was detected using Alexa Fluor 488-labeled goat anti-mouse IgG (green). (b) THP-1 cells were incubated with (center column) or without (left column) 25 μg/ml WB-6 for 2 h or 1 μM staurosporine for 6 h (right column), and expression of phosphatidylserine was tested using annexin V-biotin and fluorescein isothiocyanate (FITC)-streptavidin (green). (c) THP-1 cells were incubated with 25 μg/ml WB-6 for 2 h, and Alexa Fluor 488-labeled goat anti-mouse IgG (green) was added after (left) or before (right) fixation and permeabilization. (d) Internalization of WB-6 into HUVECs was tested according to the same protocol as (a). In all experiments, the nuclei were stained with Hoechst 33342 (blue). A representative of three independent experiments with similar results is shown.

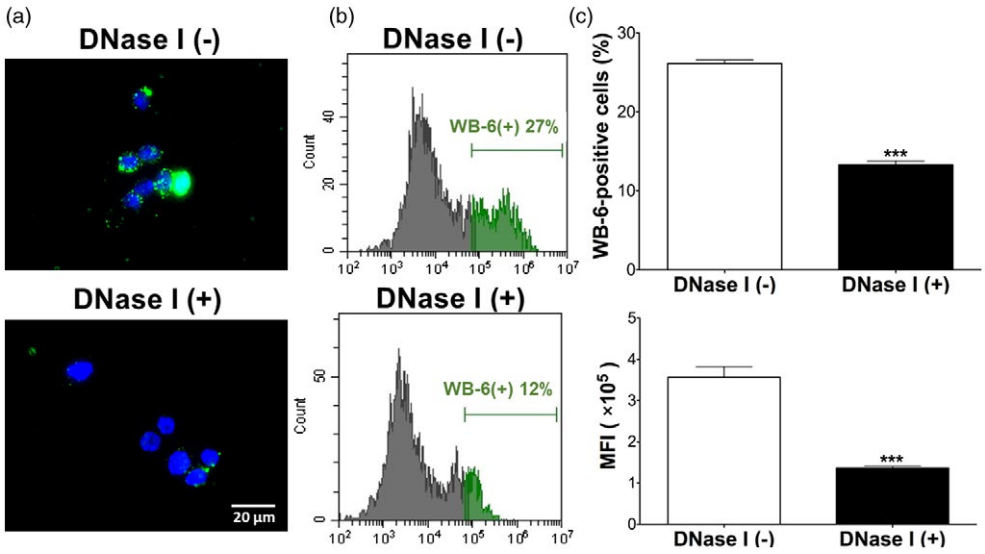

Fig. 3. Involvement of cell surface DNA in the internalization of WB-6. (a) human monocytic leukemia cell line THP-1 cells were pretreated with or without DNase 1, and internalization of WB-6 was tested as described in Fig. 2. (b) Quantitative estimation was carried out by flow cytometry and representative histograms show the ratio of Alexa Fluor 488-positive THP-1 cells with or without pretreatment with DNase 1. (c) The ratio of Alexa Fluor 488-positive THP-1 cells and mean fluorescence intensity. 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. ***$P < 0{\cdot}001$ *versus* the cells without DNase 1 pretreatment.

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-β_2GPI, 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-β_2GPI. 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-β_2GPI antibodies are known to potentially induce prothrombotic and proinflammatory states in

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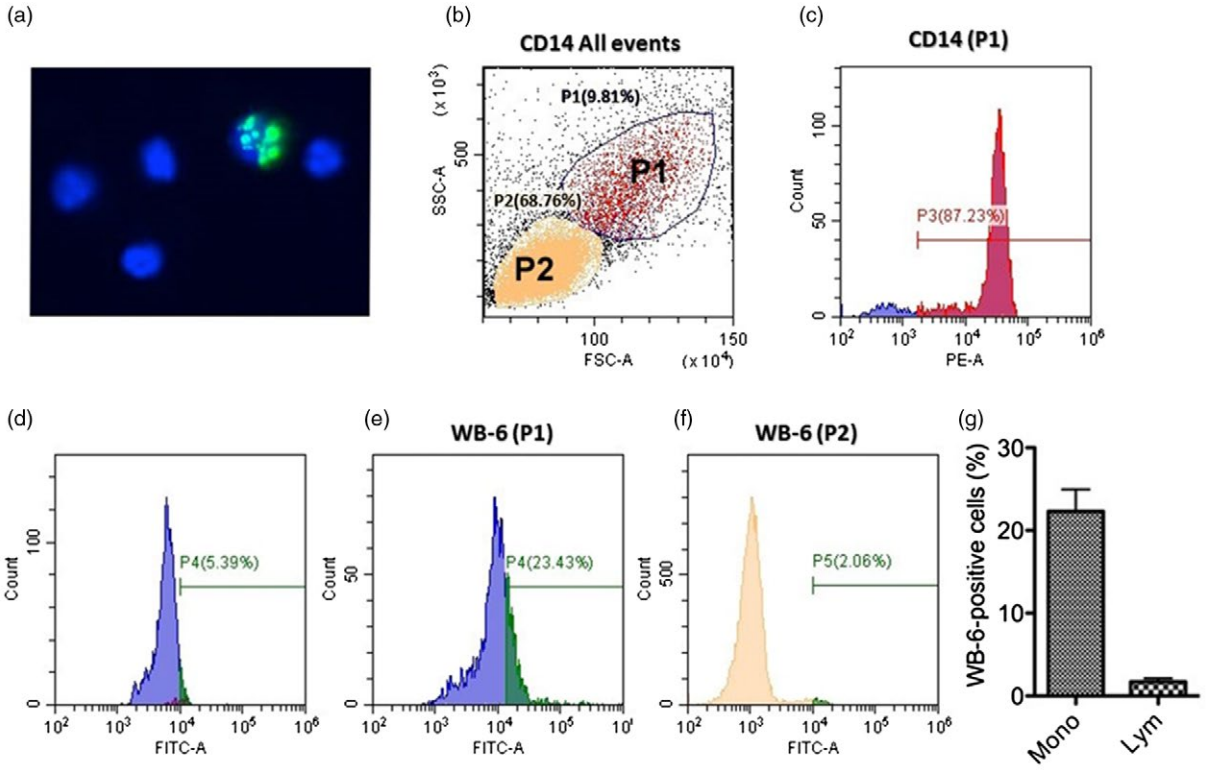

Fig. 4. Internalization of WB-6 into living peripheral blood mononuclear cells (PBMCs). (a) PBMCs from healthy volunteers were incubated with WB-6 for 2 h and internalized antibody was detected as described in Fig. 2. A representative immunofluorescence image showing that WB-6 entered only a small fraction of PBMCs. (b) In flow cytometric analysis, forward- and side-scatter plot of PBMCs showed two populations of the cells, designated P1 and P2, representing monocytes and lymphocytes, respectively. (c) Most of the cells in the P1 gate were confirmed to be $CD14^+$. (d–f) Representative histograms showing the ratio of Alexa Fluor 488-positive cells after 2 h incubation of PBMCs with isotype-matched IgG in the gate P1 (d), WB-6 in the gate P1 (e) and WB-6 in the gate P2 (f). (g) The ratio of Alexa Fluor 488-positive cells in the gate P1 (Mono) and gate P2 (Lym). 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.

monocytes and endothelial cells by binding to cell surface β_2GPI, 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-β_2GPI 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-β_2GPI in APS. From this point of view, the present finding of WB-6 dual reactivity to CL-β_2GPI 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-β_2GPI 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 β_2GPI [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

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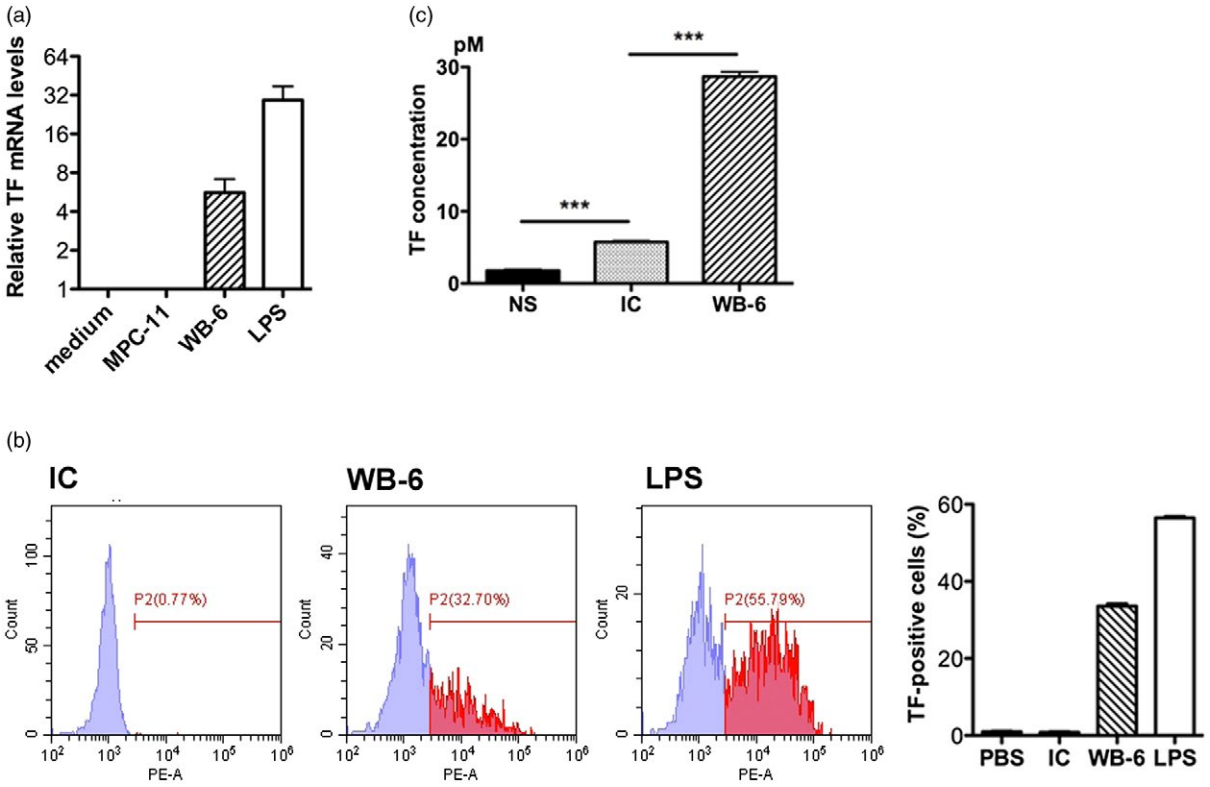

Fig. 5. Induction of tissue factor (TF) expression in monocytes by WB-6. Peripheral blood mononuclear cells (PBMCs) were incubated with WB-6, isotype-matched non-DNA-binding monoclonal antibody kappa monoclonal MPC-11, isotype-matched control immunoglobulin (Ig)G (IC) or lipopolysaccharide (LPS). (a) 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. ***$P < 0{\cdot}001$.

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 β_2GPI or DNA) were internalized by monocytic 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-β_2GPI 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

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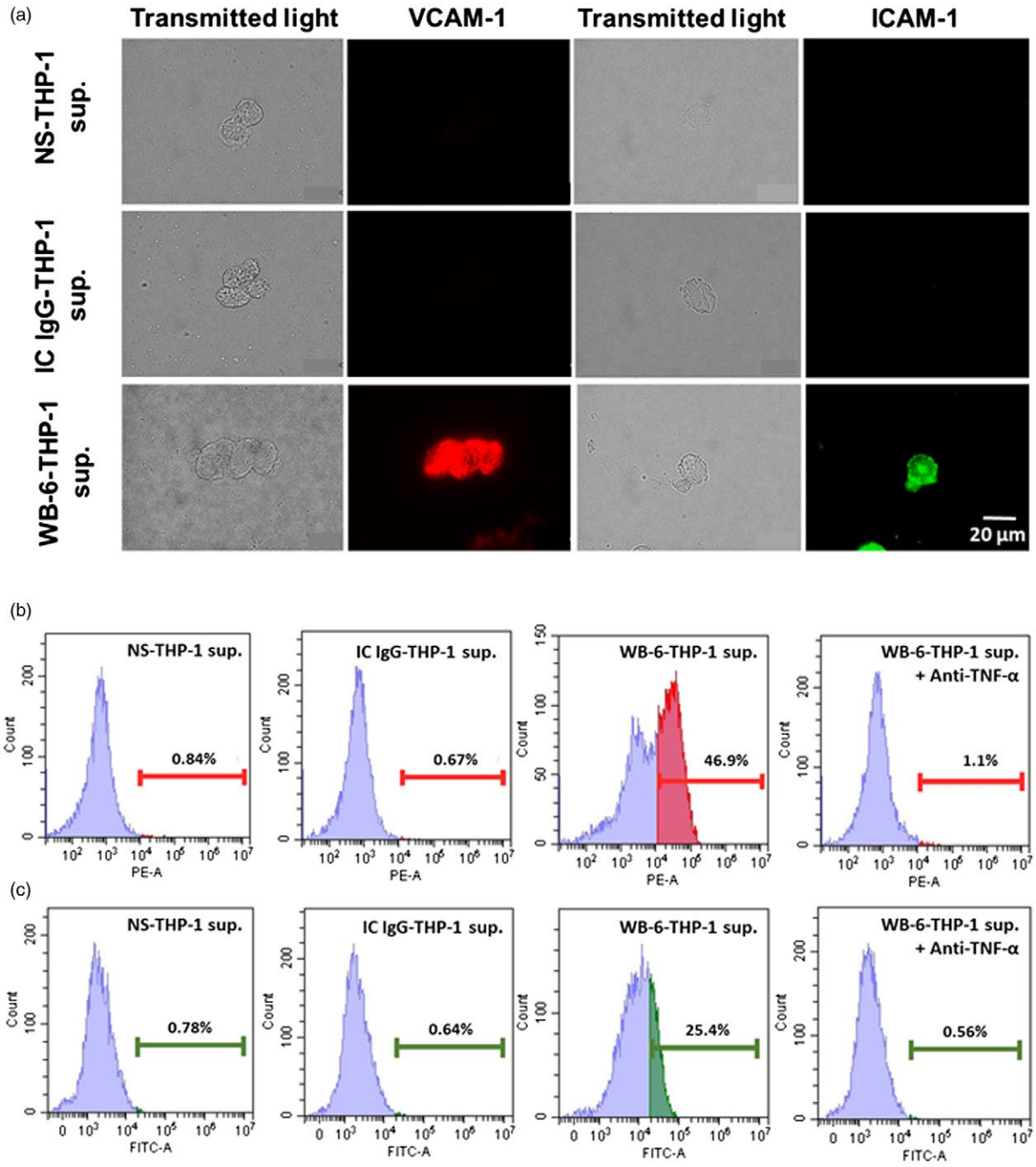

Fig. 6. Activation of human umbilical vein endothelial cells (HUVECs) by the supernatants of human monocytic leukemia cell line THP-1 cells cultured with WB-6. HUVECs were incubated for 6 h with the supernatants of THP-1 cells, which had been cultured for 4 h with/without 25 μg/ml WB-6 or isotype-matched control immunoglobulin G (IC IgG). (a) Expression of adhesion molecules was examined using phycoerythrin (PE)-labeled anti-vascular cell adhesion molecule 1 (VCAM-1) (red) and fluorescein isothiocyanate (FITC)-labeled anti- intercellular adhesion molecule 1 (ICAM-I) (green) antibodies by immunocytochemistry. Expression of VCAM-1 (b) and ICAM-1 (c) and their inhibition by anti-tumor necrosis factor (TNF)-α monoclonal antibody were estimated by flow cytometry. Data show a representative of three independent experiments with similar results; n.s. = non-stimulated.

current observations provide a new focus of interest on the role of anti-phospholipid antibodies in APS pathogenesis.

Treatment of THP-1 cells or HUVECs with DNase I significantly reduced the internalization of WB-6, but this was not complete. There could be several

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Table 1. Cytokines/chemokines in the culture supernatants of human monocytic leukemia cell line THP-1 cells incubated with isotype-matched control immunoglobulin (Ig)G or WB-6

Analyte	Control IgG (pg/ml)	WB-6 (pg/ml)
IL-1β	<1·82	<1·82
IFN-α2	<1·51	<1·51
IFN-γ	<1·65	<1·65
TNF-α	<2·14	111
MCP-1	27·4	424
IL-6	<2·30	<2·30
IL-8	8·80	617
IL-10	<1·51	<1·51
IL-12p70	<1·47	<1·47
IL-17A	<0·96	<0·96
IL-18	<2·22	<2·22
IL-23	3·69	5·33
IL-33	<9·17	<9·17

IL = interleukin; IFN = interferon; TNF = tumor necrosis factor; MCP-1 = monocyte chemoattractant protein-1.

explanations for these results, including that cell surface DNA was not completely removed by DNase I in our assay, or that small amounts of DNA were attached to the WB-6 antibody preparation itself. WB-6 may also bind to cell surface β_2GPI if it exists, but whether this leads to internalization remains unclear. One molecule of β_2GPI can be bound by one molecule of WB-6, but in contrast a comparable molecular mass of DNA can be bound by many WB-6 antibody molecules. This could result in the formation of large immune complexes which may trigger endocytosis mechanisms, leading to internalization of WB-6 together with the DNA. There is a report in the literature documenting the internalization of FITC-labeled β_2GPI into the cytoplasm [33], but this used Chinese hamster ovary cells over-expressing TLR-4, and may not reflect the natural setting.

Which signaling pathway(s) were activated by the internalization of WB-6 is a challenging issue which remains to be clarified. As a result of intensive studies in the field of innate immunity, it has recently been discovered that not only bacterial DNA but also mammalian DNA can be recognized by cytoplasmic sensors and thereby activate signaling pathways. For example, absent in melanoma 2 (AIM2) has been reported to sense cytoplasmic DNA transfected using liposomes, leading to activation of the ASC pyroptosome and caspase-1 and secretion of IL-1β [34]. Murine embryonic fibroblasts expressing stimulator of interferon genes (STING) homodimers were able to bind to transfected DNA, leading to TNF-α production [35]. While information on the sensor which plays a major role in detecting DNA-anti-DNA immune complexes is very limited, human DNA has been reported to induce IL-1β 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].

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 β_2GPI, 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-β_2GPI antibodies bind β_2GPI 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 β_2GPI, nor the co-receptor TLR-4, and therefore β_2GPI 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-β_2GPI-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 β_2GPI 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

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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-β_2GPI 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-β_2GPI 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-I and VCAM-I. 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-Myd88 pathway. A subset of anti-phospholipid antibodies with these characteristics, whether they are monoclonal antibodies with dual-reactivity or polyclonal mixture of anti-β_2GPI 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.

References

1 Levine JS, Branch DW, Rauch J. The antiphospholipid syndrome. N Engl J Med 2002; **346**:752–63.

2 Andreoli L, Chighizola CB, Banzato A *et al*. Estimated frequency of antiphospholipid antibodies in patients with pregnancy morbidity, stroke, myocardial infarction, and deep vein thrombosis: a critical review of the literature. Arthritis Care Res 2013; **65**:1869–73.

3 Cervera R, Boffa MC, Khamashta MA, Hughes GRV. The Euro-Phospholipid project: epidemiology of the antiphospholipid syndrome in Europe. Lupus 2009; **18**:889–93.

4 de Laat HB, Derksen RHWM, Urbanus RT *et al*. β_2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood 2004; **104**:3598–602.

5 Nishimura M, Nii T, Trimova G *et al*. The NF-kappaB specific inhibitor DHMEQ prevents thrombus formation in a mouse model of antiphospholipid syndrome. J Nephropathol 2013; **2**:114–21.

6 Ağar Ç, van Os GMA, Morgelin M *et al*. β_2-glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. Blood 2010; **116**:1336–43.

7 Mahler M, Norman GL, Meroni PL, Khamashta M. Autoantibodies to domain 1 of beta 2 glycoprotein 1: a promising candidate biomarker for risk management in antiphospholipid syndrome. Autoimmun Rev 2012; **12**:313–7.

8 Amengual O, Atsumi T, Oku K *et al*. Phospholipid scramblase 1 expression is enhanced in patients with antiphospholipid syndrome. Mod Rheumatol 2013; **23**:81–8.

9 Meroni PL, Borghi MO, Raschi E, Tedesco F. Pathogenesis of antiphospholipid syndrome: understanding the antibodies. Nat Rev Rheumatol 2011; **7**:330–9.

10 Bai A. β_2-glycoprotein I and its antibodies involve in the pathogenesis of the antiphospholipid syndrome. Immunol Lett 2017; **186**:15–9.

11 Sorice M, Longo A, Capozzi A *et al*. Anti- β_2-glycoprotein I antibodies induce monocyte release of tumor necrosis factor α and tissue factor by signal transduction pathways involving lipid rafts. Arthritis Rheum 2007; **56**:2687–97.

12 Zhou H, Yan Y, Xu G *et al*. Toll-like receptor (TLR)-4 mediates anti-β_2GPI/ β_2GPI-induced tissue factor expression in THP-1 cells. Clin Exp Immunol 2011; **163**:189–98.

13 Xie H, Sheng L, Zhou H, Yan J. The role of TLR4 in pathophysiology of antiphospholipid syndrome-associated thrombosis and pregnancy morbidity. Br J Haematol 2014; **164**:165–76.

14 Agostinis C, Biffi S, Garrovo C *et al*. *In vivo* distribution of β_2 glycoprotein I under various pathophysiologic conditions. Blood 2011; **118**:4231–8.

15 Laplante P, Amireault P, Subang R *et al*. Interaction of β_2-glycoprotein I with lipopolysaccharide leads to Toll-like receptor 4 (TLR4)-dependent activation of macrophages. J Biol Chem 2011; **286**:42494–503.

16 Kubota T, Watanabe N, Kanai Y, Stollar BD. Enhancement of oxidative cleavage of DNA by the binding sites of two

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anti-double-stranded DNA antibodies. J Biol Chem 1996; **271**:6555–61.

17 Koutouzov S, Cabrespines A, Amoura Z *et al.* Binding of nucleosomes to a cell surface receptor: redistribution and endocytosis in the presence of lupus antibodies. Eur J Immunol 1996; **26**:472–86.

18 Laktionov PP, Tamkovich SN, Rykova EY *et al.* Cell-surface-bound nucleic acids: free and cell-surface-bound nucleic acids in blood of healthy donors and breast cancer patients. Ann NY Acad Sci 2004; **1022**:221–7.

19 Pierangeli SS, Espinola RG, Liu X, Harris EN. Thrombogenic effects of antiphospholipid antibodies are mediated by intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and P-selectin. Circ Res 2001; **88**:245–50.

20 Vega-Ostertag M, Casper K, Swerlick R *et al.* Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. Arthritis Rheum 2005; **52**:1545–54.

21 Lafer EM, Rauch J, Andrzejewski C *et al.* Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. J Exp Med 1981; **153**:897–909.

22 Cocca BA, Seal SN, D'Agnillo P *et al.* Structural basis for autoantibody recognition of phosphatidylserine-β2 glycoprotein I and apoptotic cells. Proc Natl Acad Sci USA 2001; **98**:13826–31.

23 Kumar S, Nagl S, Kalsi JK *et al.* Anti-cardiolipin/β-2 glycoprotein activities co-exist on human anti-DNA antibody light chains. Mol Immunol 2003; **40**:517–30.

24 Alarcon-Segovia D, Ruiz-Arguelles A, Fishbein E. Antibody to nuclear ribonucleoprotein penetrates live human mononuclear cells through Fc receptors. Nature 1978; **271**:67–9.

25 Zannikou M, Bellou S, Eliades P *et al.* DNA-histone complexes as ligands amplify cell penetration and nuclear targeting of anti-DNA antibodies via energy-independent mechanisms. Immunology 2016; **147**:73–81.

26 Yanase K, Smith RM, Puccetti A *et al.* Receptor-mediated cellular entry of nuclear localizing anti-DNA antibodies via myosin 1. J Clin Invest 1997; **100**:25–31.

27 Jang JY, Jeong JG, Jun HR *et al.* A nucleic acid-hydrolyzing antibody penetrates into cells via caveolae-mediated endocytosis, localizes in the cytosol and exhibits cytotoxicity. Cell Mol Life Sci 2009; **66**:1985–97.

28 Rivadeneyra-Espinoza L, Ruiz-Argüelles A. Cell-penetrating anti-native DNA antibodies trigger apoptosis through both the neglect and programmed pathways. J Autoimmun 2006; **26**:52–6.

29 Portales-Perez D, Alarcón-Segovia D, Llorente L *et al.* Penetrating anti-DNA monoclonal antibodies induce activation of human peripheral blood mononuclear cells. J Autoimmun 1998; **11**:563–71.

30 Jang EJ, Nahm DH, Jang YJ. Mouse monoclonal autoantibodies penetrate mouse macrophage cells and stimulate NF-κB activation and TNF-α release. Immunol Lett 2009; **124**:70–6.

31 Prinz N, Clemens N, Strand D *et al.* Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. Blood 2011; **118**:2322–32.

32 Viall CA, Chen Q, Liu B *et al.* Antiphospholipid antibodies internalised by human syncytiotrophoblast cause aberrant cell death and the release of necrotic trophoblast debris. J Autoimmun 2013; **47**:45–57.

33 Raschi E, Chighizola CB, Grossi C *et al.* β2-glycoprotein I, lipopolysaccharide and endothelial TLR4: three players in the two hit theory for anti-phospholipid-mediated thrombosis. J Autoimmun 2014; **55**:42–50.

34 Fernandes-Alnemri T, Yu JW, Datta P *et al.* AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 2009; **458**:509–13.

35 Abe T, Harashima A, Xia T *et al.* STING recognition of cytoplasmic DNA instigates cellular defense. Mol Cell 2013; **50**:5–15.

36 Shin MS, Kang Y, Lee N *et al.* Self double-stranded (ds)DNA induces IL-1β production from human monocytes by activating NLRP3 inflammasome in the presence of anti-dsDNA antibodies. J Immunol 2013; **190**:1407–15.

37 Schecter AD, Rollins BJ, Zhang YJ *et al.* Tissue factor is induced by monocyte chemoattractant protein-1 in human aortic smooth muscle and THP-1 cells. J Biol Chem 1997; **272**:28568–73.

38 Kubota T, Fukuya Y, Hashimoto R *et al.* Possible involvement of chemokine-induced platelet activation in thrombophilic diathesis of antiphospholipid syndrome. An attractive target for the NF-κB-specific inhibitor DHMEQ. Ann NY Acad Sci 2009; **1173**:137–45.

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