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The role of β_2-glycoprotein I (β_2GPI) carbohydrate chains in the reactivity of anti-β_2GPI antibodies from patients with primary antiphospholipid syndrome and in the activation and differentiation of U937 cells

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ABSTRACT

Several studies have shown that conformational changes of β_2-glycoprotein I (β_2GPI) when bound to negatively charged components expose cryptic epitopes and subsequent binding of anti-β_2GPI from patients with antiphospholipid syndrome (APS). However, the role of the carbohydrate chains of β_2GPI in this anti-β_2GPI reactivity is poorly understood. We therefore studied the reactivity and inhibition of anti-β_2GPI antibodies from APS patients with native, partially glycosylated β_2GPI (pdβ_2GPI; without sialic acid) and completely deglycosylated β_2GPI (cdβ_2GPI). To determine the potential biologic importance of these glycoforms and their interaction with anti-β_2GPI *in vitro*, stimulation assays were performed with the U937 cell line. Circular dichroism (CD) and fluorescence analysis of the three β_2GPI forms were also studied. We found an increased reactivity of anti-β_2GPI against pdβ_2GPI and cdβ_2GPI compared to native β_2GPI. Both deglycosylated β_2GPI isoforms showed higher inhibition of the anti-β_2GPI reactivity than the native protein in soluble-phase. Likewise, the antibody/β_2GPI/glycoform complexes increased the synthesis of IL-6, IFNγ and TNFα and the expression of HLA-DR, CD14 and CD11c in U937 cells. CD and fluorescence studies of the glycoforms yielded considerable changes in the fluorescence signals. Our work suggests that the partial or complete removal of the carbohydrate chains uncover cryptic epitopes present in β_2GPI. The differentiation and increased synthesis of pro-inflammatory cytokines by U937 cells *in vitro* may have pathogenetic implications.

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1. Introduction

Antiphospholipid antibodies (aPL) is a heterogeneous family of antibodies that react with plasma proteins, among which β_2-glycoprotein I (β_2GPI) has been shown to be the major target antigen for aPL of patients with APS [1–5]. β_2GPI, a glycoprotein of 50 kDa, contains four N-linked carbohydrate side chains that account for approximately 15% (wt/wt) of its total molecular mass, consisting of 326 amino acids residues organized in five highly conserved subunits, called complement control protein domains or "*sushi*" domains [6–8]. Each domain is comprised of 60 amino acid residues, except for domain V that has 80 amino acid residues due to a C-terminal extension of 19 amino acids and an insertion of six amino acids, forming a hydrophobic loop. Domain V contains a region rich in lysine residues 280–288 which is the binding site of the negatively charged compounds (e.g., PLs, DNA, low density lipoprotein, heparin) and platelets factor 4, apolipoprotein E receptor 2, glycoprotein Ibα, annexin II, TLR-2 and TLR-4 [9–15].

Although the function of β_2GPI is unknown, it has been implicated in several regulatory functions in coagulation [13,16–20], displacement of the annexin-V attached to cell membranes and removal of apoptotic bodies [21,22]. In addition anti-β_2GPI/β_2GPI complexes are also capable of activating platelets and endothelial cells [23].

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Studies of the interaction between β_2GPI and PL have established that the binding of β_2GPI to PL of platelets and endothelial cell membranes induces a conformational change in the β_2GPI, thus allowing the exposure of cryptic epitopes present in domain I, the main epitope recognized by pathogenic anti-β_2GPI [24,25]. The interaction of the anti-β_2GPI/β_2GPI/PL complex in genetically susceptible individuals disturbs the homeostatic reactions by the activation of the vascular endothelium and platelets [12,13,23,26]. In endothelial cells there is an increased expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin [14,27], in addition to allowing the expression of tissue factor [28] and the production of proinflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α and MCP-1 perpetuating activation and inducing a prothrombotic state [29,30].

It is currently thought that β_2GPI carbohydrates are only involved in hiding a cryptic epitope localized mainly in domain I (G40-R43) [24]. de Laat et al. first put this hypothesis forward, when they described that the reactivity of anti-β_2GPI against epitope G40-R43 increases when carbohydrates are removed [24]. Furthermore, recognition showed a strong association with thrombosis in patients with APS [30]. Meanwhile, Kondo showed that β_2GPI of patients with APS have less sialic acid in carbohydrates antennae (N143 and N174), leading to lessened interaction of the negative charges of sialic acid with the G40-R43 epitope [31], if this allows the exposure of neo-epitopes is unknown. In the present study, we report that carbohydrates are essential for covering cryptic epitopes present in the β_2GPI, and that they prevent the reactivity of anti-β_2GPI with β_2GPI in liquid form. We also found that the loss of sialic acid is sufficient for the activation and differentiation of the U937 cell line induced by anti-β_2GPI–β_2GPI complexes.

2. Methodology

2.1. Patients and controls

We included patients that fulfilled the Sydney and/or Alarcón-Segovia criteria for the classification of primary antiphospholipid syndrome (PAPS) [32,33]. Patients that met criteria for the classification of SLE or positive anti-DNA antibodies were excluded [34]. Sera of healthy individuals were studied as controls.

2.2. Purification of human β_2GPI

Purification of β_2GPI was performed as previously described [16] with some minor modifications. The proteins in heparin column were eluted with several salt solutions [(1) 20 mM Tris–HCl, 30 mM NaCl; (2) 20 mM Tris–HCl, 150 mM NaCl and (3) 20 mM Tris–HCl, 1 M NaCl], and the second elution fraction was recovered and passed through an immunoaffinity column of anti-β_2GPI antibodies.

2.3. Purification of IgG anti-β_2GPI antibodies

This was done in a spin column of protein A-Sepharose (SIGMA. St. Louis, MO, USA) with patients serum diluted in PBS at a final concentration of 1 mg/ml.

2.4. Generation of β_2GPI glycoforms

Enzymatic Protein Deglycosylation Kit (SIGMA) was used for partial deglycosylation with neuraminidase as the only enzyme present in the reaction. *GlycoProfile IV Chemical Deglycosylation Kit* (SIGMA) was used to complete deglycosylation, according to the manufacturer's recommendations. The product reactions were subsequently ran through a heparin column as described above.

2.5. Spectroscopic measurements of glycoforms

Far-UV CD spectra were recorded on a JASCO J-720 spectropolarimeter, as described elsewhere. CD signals are reported as mean residue ellipticity, (θ), using a value of 110 for the molecular weight of a mean residue. Thermal melting profiles were obtained at a heating rate of 1 °C/min, following the ellipticity change at 222 nm. Fluorescence spectra were recorded in an Olis DM45 scanning spectrofluorometer.

2.6. Reactivity against β_2GPI and cardiolipin

A commercial ELISA kit QUANTA Lite equipment system (INOVA Diagnostic, San Diego, CA, USA) was used to determine aCL (IgG) and anti-β_2GPI (IgG) antibodies during clinical antibody screening. Results are reported in UGPL units. All assays were performed according to the manufacturer's specifications. The results were evaluated using a cut-off obtained by 95th percentile.

2.7. Anti-β_2GP-I ELISAs against native and β_2GPI glycoforms and CL-glycoforms complexes

These were performed as previously described in detail [35] with slight modifications. We utilized 96 wells polystyrene plates (NALGE Nunc. Naperville, IL, USA) and were sensitized with 100 µl of a 10 µg/mL solution of native or β_2GPI glycoforms.

2.8. Inhibition assays against native and β_2GPI glycoforms

Purified IgG was added to different concentrations of β_2GPI, dpβ_2GPI, dcβ_2GPI or BSA [0, 3, 5, 10, 15 and 20 µg/mL] and the reactions were incubated overnight at room temperature. The ELISA's were then performed as described above in plates sensitized with native or β_2GPI glycoforms. Percent inhibition was calculated as follows:

$$\%\ \text{inhibition} = [(\text{OD anti-}\beta_2\text{GPI} - \text{OD anti-}\beta_2\text{GPI with inhibitor})/\text{OD anti-}\beta_2\text{GPI}] \times 100$$

2.9. Cell differentiation markers and measurement of cytokines from active U937 culture with glycoforms-antibodies complexes

5×10^5 U937 cells were cultured in 24-well plates with RPMI 1640 supplemented medium (GIBCO, Life technology, Grand Island, NY, USA). Cells were washed with PBS and Incubated in medium serum-free with native β_2GPI and glycoforms for 2 h [final concentration of 10 µg/mL]. Subsequently, purified IgG was added and incubated for 6 h. The cells and supernatant were collected for the measurement of differentiation and cytokine release studies, as described below.

2.10. Quantification of IL-6, IL-4, IFNγ and TNFα

This was performed with a kit *Human Cytokine* (Millipore Corp. Billerica, MA, USA), according to the manufacturer's specifications. The plate was read in *Lincoplex 200* with the software *Luminex 100 IS 2.3* software.

2.11. Differentiation flow cytometric studies

We utilized anti-CD14-FITC, anti-HLA-DR-PE and anti-CD11c-PE for flow cytometry and analyzed them with BD Accuri C6 flow cytometer (BD Accuri C6 flow cytometer, Piscataway, NJ, USA).

2.12. Statistical analysis

All statistical analyses were performed using GraphPad 5.0 software (GraphPad software, Inc., La Jolla, CA, USA) The Kruskal–Wallis test and Mann–Whitney *U* test were used to determine differences between groups. All tests were 2-sided statistical analyses and *p*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Patients

We studied 24 patients with PAPS (15 women), age 29 ± 9.8 years and 9.5 ± 5.5 years of disease duration. Ten healthy subjects (7 females) were studied as controls. 86% of the PAPS sera were positive for aCL IgG antibodies, while 96% were positive for IgG antibodies as determined by commercial ELISA aCL anti-β_2GPI assays [Fig. 1A].

3.2. Partial and complete deglycosylation of β_2GPI

We corroborated the results of the enzymatic reactions by the electrophoretic mobility of native β_2GPI, pdβ_2GPI and cdβ_2GPI isoforms. These results are shown in Fig. 1B.

3.3. Far-UV circular dichroism and fluorescence studies of native and β_2GPI glycoforms

To determine whether the deglycosylation process changed the tertiary structure of β_2GPI, far-UV CD and fluorescence were performed. As seen in Fig. 1C and D, pdβ_2GPI exhibited a CD spectrum with some modest variations in relation to that of native β_2GPI. In contrast, cdβ_2GPI yielded large differences in the CD signals.

3.4. Reactivity of purified IgG antibodies against native β_2GPI, pdβ_2GPI and cdβ_2GPI

We determined the reactivity of purified IgG from PAPS patients and healthy subjects against native β_2GPI and its two glycoforms by ELISA in non-irradiated (neutral) plates. We found that the

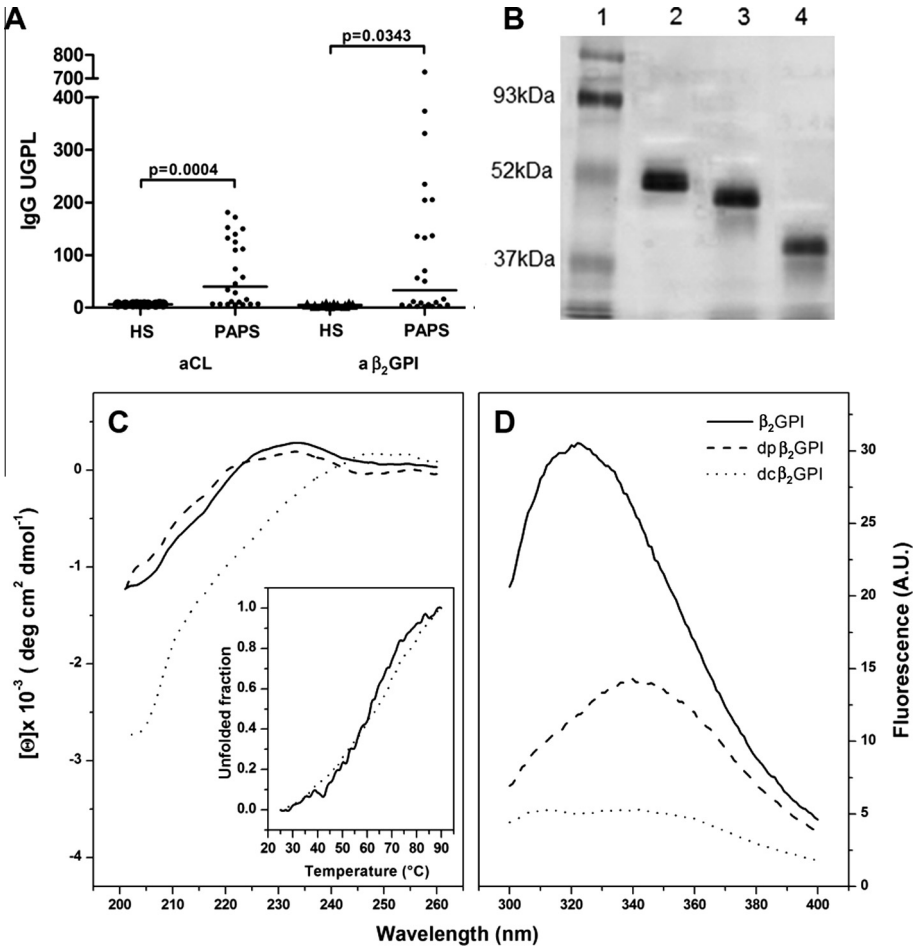

Fig. 1. (A) Reactivity of sera from 24 patients with primary anti-phospholipid syndrome (PAPS) and 10 healthy subjects (HS) detected with a commercial aCL and anti-β2GPI kit. Numbers represent median UGPL. (B) Electrophoretic shift of glycoforms of β2GPI: Lane 1 = MW; lane 2 = β2GPI native (50 kDa); lane 3 = pdβ2GPI (47 kDa) and lane 4 = cdβ2GPI (38 kDa). (C) Spectroscopic properties of β2GPI and its deglycosylated forms. All spectra were recorded using a protein concentration of ~3 μM, in PB buffer, at 25 °C. Far-UV CD spectra. Inset. Thermal melting profiles obtained following the CD signal at 222 nm, at a scan rate of 1 °C/min. The apparent unfolded fraction was obtained by normalizing the CD signal at any temperature in relation to the difference in ellipticity at 25 and 90 °C. (D) Fluorescence spectra, using an excitation wavelength of 290 nm.

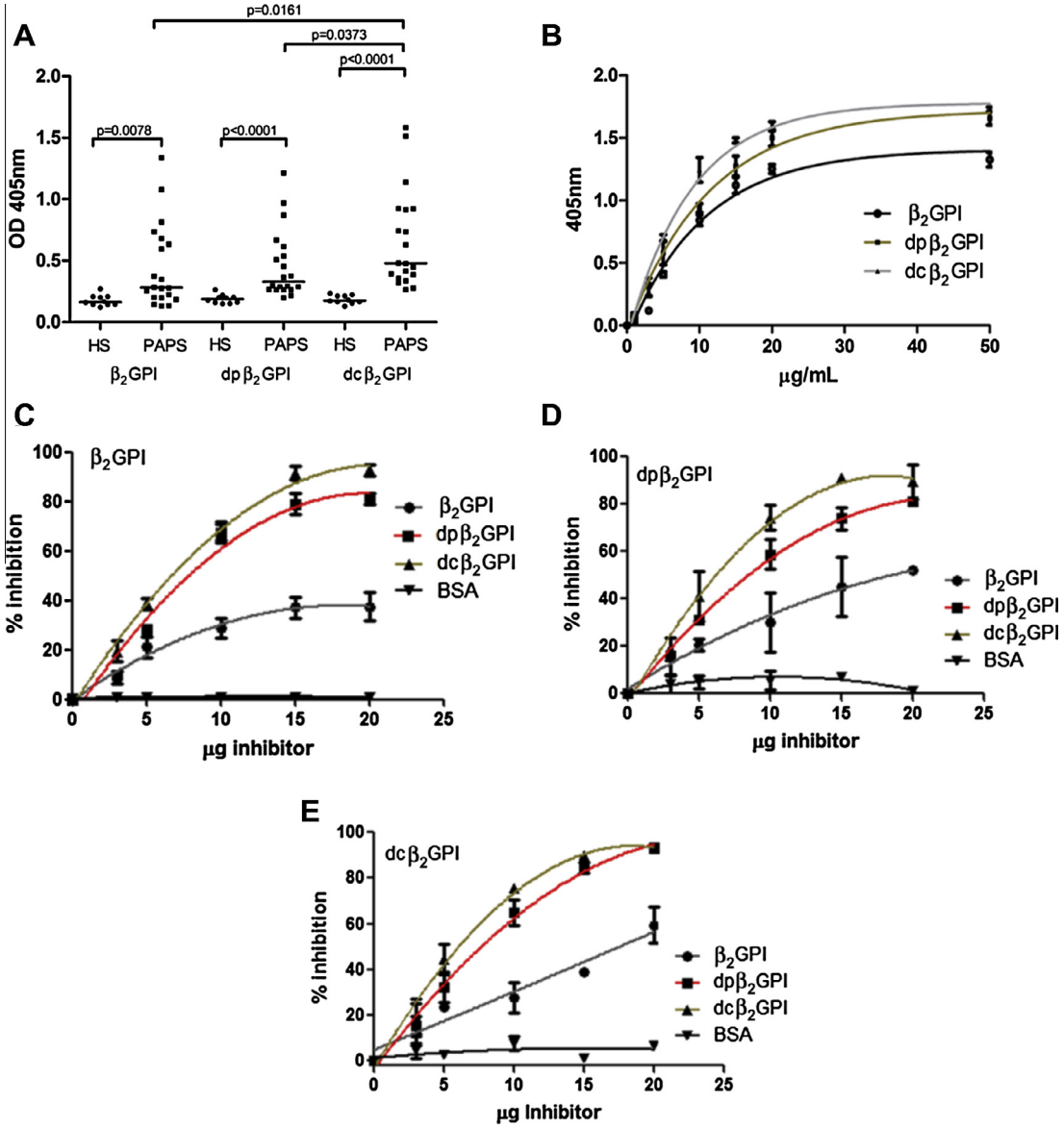

Fig. 2. (A) Reactivity of IgG purified antibodies from 24 patients with PAPS and 10 healthy subjects in plates sensitized with β2GPI, pdβ2GPI y cdβ2GPI. Data are represented as median, with significance value of $p < 0.05$. (B) Dose–response curves of 3 samples of PAPS IgG antibodies against β2GPI, pdβ2GPI and cdβ2GPI. (C–E) Inhibition assays of IgG antibodies from patients with PAPS using native β2GPI, pdβ2GPI and cdβ2GPI or BSA as inhibitors in non-irradiated plates sensitized with: (C) native β2GPI; (D) pdβ2GPI or (E) cdβ2GPI. Results represent mean ± SEM.

reactivity of PAPS IgG against native β_2GPI, dpβ_2GPI, and dcβ_2GPI was 1.7-fold, 1.75-fold and 2.7-fold higher compared with normal IgG, respectively [Fig. 2A]. When we compared the reactivity of IgG anti-β_2GPI against cdβ_2GPI, we found a 1.4-fold and 1.7-fold increase reactivity compared to pdβ_2GPI and native β_2GPI antigens, respectively [Fig. 2A]. No differences were found in antibody reactivity between dpβ_2GPI and β_2GPI [Fig. 2A]. The IgG anti-β_2GPI reactivity against the native and modified antigens followed a dose–response curve (Fig. 2B). We found no differences in reactivity of the IgG antibodies from patients with PAPS in non-irradiated plates. The reactivity of anti-β_2GPI studied in non-irradiated plates sensitized with CL-β_2GPI, CL-pdβ_2GPI or CL-cdβ_2GPI yielded similar results (data not shown).

3.5. Inhibition of IgG anti-β_2GPI by liquid-phase β_2GPI, pdβ_2GPI or cdβ_2GPI

We determined the inhibitory capacity of the different modifications of the β_2GPI by crossed-inhibitory assays. We found that cdβ_2GPI and pdβ_2GPI inhibited more than 80% of the antibody reactivity against the three β_2GPI antigens in a dose–response fashion and that they reached a plateau approximately at 15 μg/mL (Fig. 2C–E). In the same figures it can be seen that native β_2GPI inhibited only about 40% of IgG antibodies reactive with native β_2GPI and the 2 β_2GPI glycoforms.

3.6. Expression of U937differentiation markers

We determined whether anti-β_2GPI antibodies complexes are capable of differentiating U937 cells into monocytes. For this, U937 cells were preincubated with native β_2GPI and the two β_2GPI glycoforms and subsequently with purified normal human IgG or IgG aPL. As seen in Fig. 3, an increase in the expression of CD14, CD11c and HLA-DR was obtained when stimulated with aPL–pdβ_2GPI or aPL–cdβ_2GPI complex, while the aPL in the presence of native β_2GPI only induced a slight increase of these differentiation markers. No expression of CD14, CD11c and HLA-DR was seen in cells stimulated with aPL alone [Fig. 3].

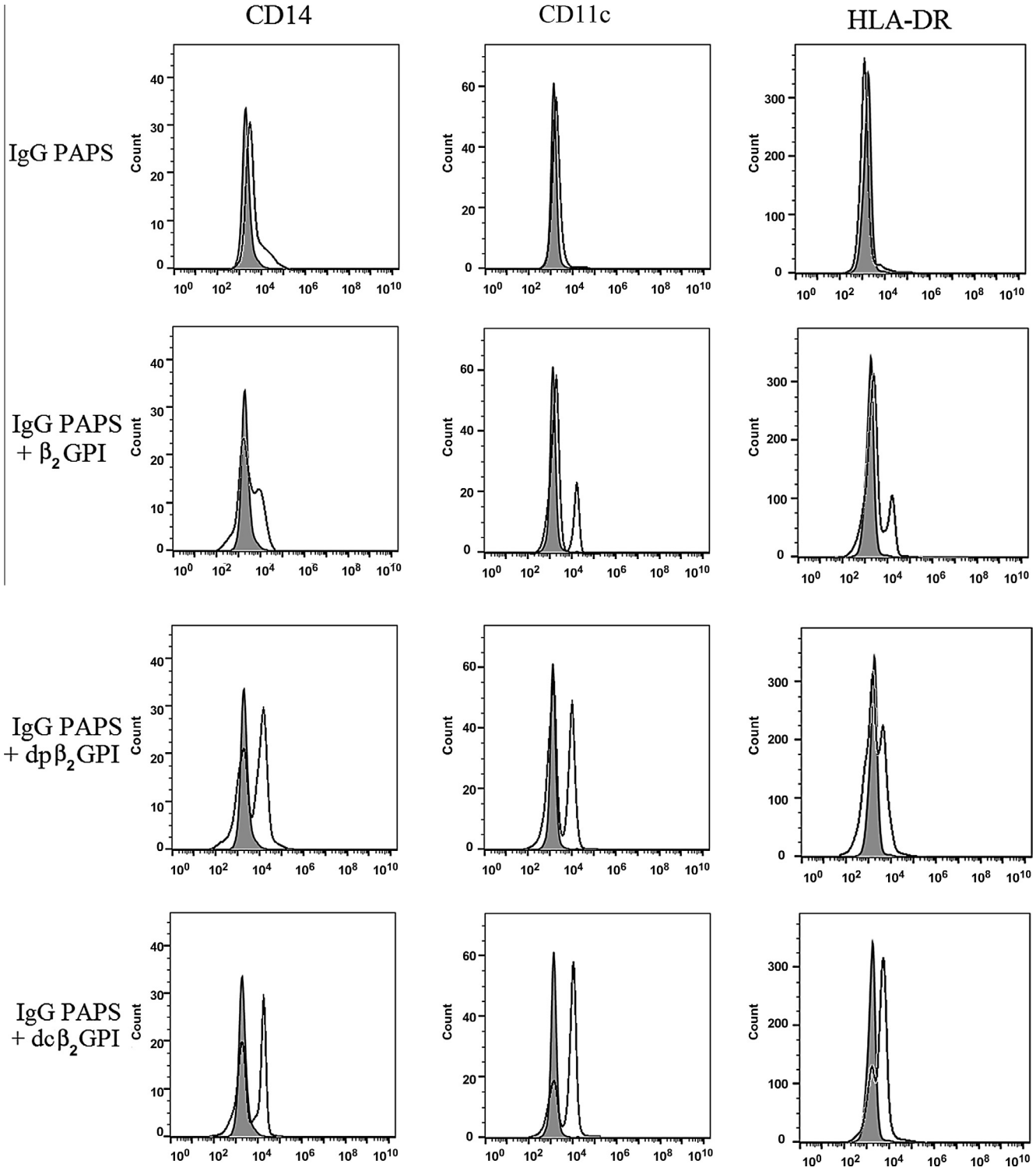

Fig. 3. Expression of CD14, CD11c and HLA-DR in U937 cells after 6 h of stimulation with IgG aPL, IgG aPL–β2GPI, IgG aPL–pdβ2GPI or IgG aPL–cdβ2GPI complexes.

3.7. Synthesis of pro-inflammatory cytokines by U937 cells induced by IgG APS

We studied whether the different anti-β_2GPI-antigen complexes are capable of stimulating the synthesis of cytokines in a multiple detection assay. The results of these experiments are shown in Fig. 4A–C. Interestingly, except for TNFα, PAPS IgG greatly enhanced the synthesis of IL-6 and IFNγ compared to normal human IgG in the absence of exogenous human β_2GPI. The same figures show that the synthesis of the three cytokines was further increased by PAPS IgG when cells were preincubated with native β_2GPI, pdβ_2GPI or cdβ_2GPI. No synthesis of IL-4 was found (data not shown).

4. Discussion

Several groups, including ours, have shown that binding of β_2GPI to cell membranes or negatively charged surfaces induces

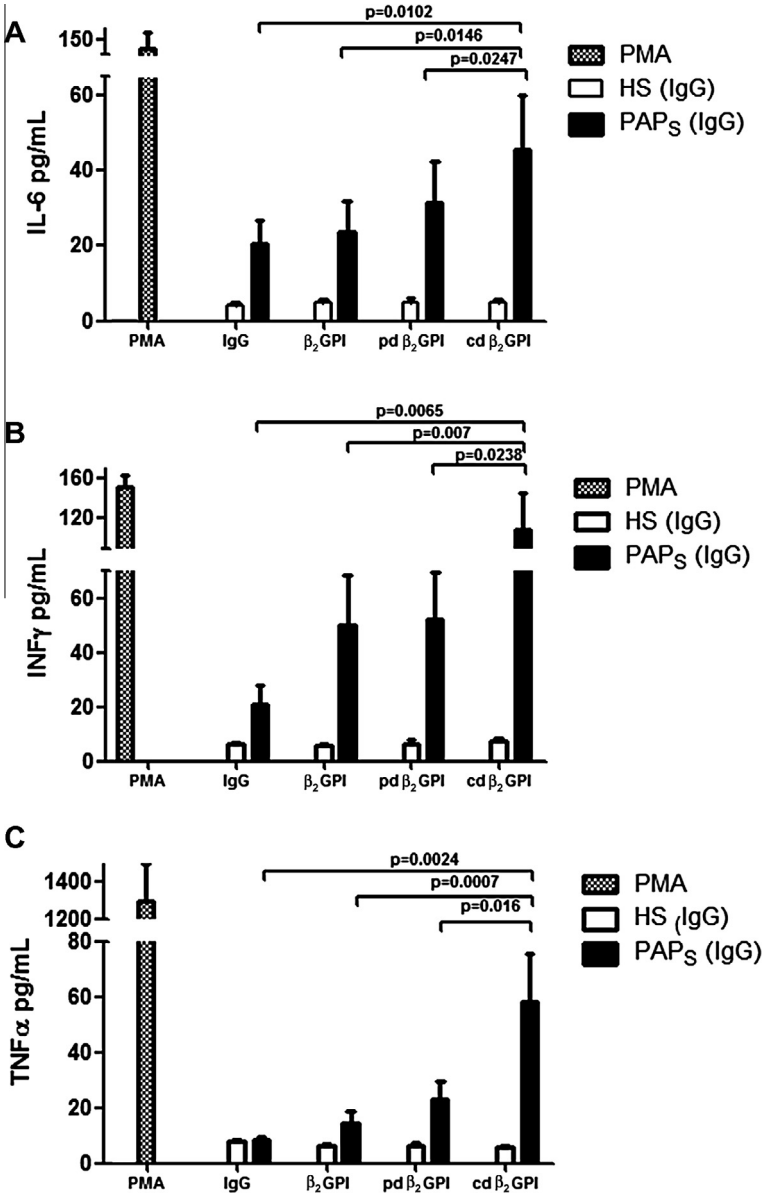

Fig. 4. Concentration of pro-inflammatory cytokines in culture supernatants of U937 cells after 6 h of stimulation with IgG aPL–β2GPI, IgG aPL–pdβ2GPI and IgG aPL–cdβ2GPI complexes from 11 patients with PAPS and 10 healthy subjects, (A) IL-6, (B) IFNγ, (C) TNFα. Basal synthesis of the three cytokines was below the level of detection (3.2 pg/ml). Results are expressed as mean ± SEM. Values $p < 0.05$ are significant.

conformational changes in the β_2GPI exposing cryptic epitopes and subsequent reactivity of anti-β_2GPI antibodies [4,25,36,37]. This epitope exposure has been explained mostly by changes in the makeup of β_2GPI from a circular to a J-form, thus allowing the exposure of epitope G40-R43 in Domain I and the subsequent recognition by anti-β_2GPI antibodies [24,30,38]. Kondo et al. reported that some APS patients have β_2GPI molecules with a reduced number of negatively charged sialic acid units at Asn-143, suggesting a conformational protein instability perhaps rendering it more immunogenic [31]. Here, we showed that the removal of the sialic acid is sufficient to expose cryptic epitopes and increase the reactivity of anti-β_2GPI relative to that of native β_2GPI. This finding offers a possible explanation of the potential binding capacity of anti-β_2GPI to an abnormally desialyted β_2GPI *in vivo* [31]. Our work also showed an increased anti-β_2GPI binding after the complete removal of its carbohydrate chains, consistent with de Laat et al. [24]. An interesting finding reported here is that although the deglycosylated isoforms have approximately twofold capacity than native β_2GPI to inhibit IgG anti-β_2GP-I in soluble-phase, native β_2GPI also inhibited IgG anti-β_2GPI under the same *in vitro* conditions in a dose response manner. These results are in agreement with our group's earlier proposal regarding the existence of both cryptic and non-cryptic epitopes present in β_2GPI [39]. We also found that carbohydrates are a determining factor of β_2GPI conformation and that in spite of the dramatic changes in its spectral properties, cdβ_2GPI seems to preserve a highly folded conformation of unknown shape.

Our work also showed for the first time that IgG aPL induce differentiation of U937 cells into monocytes and synthesis of proinflammatory cytokines in the presence of pd β_2GPI, cd β_2GPI or native β_2GPI. We also found that these synthetic capacities were significantly higher when cells were preincubated with cd β_2GP-I compared to pd β_2GPI and native β_2GPI. These results suggest that the conformational changes acquired by β_2GPI after complete deglycosylation render it more amenable to cell membrane anchorage of an already opened protein for anti-β_2GPI reactivity.

Interestingly, the finding described here demonstrates that PAPS IgG stimulates high cytokine synthesis compared to normal IgG in the absence of added exogenous β_2GPI. Because cells were incubated in serum-free medium 2 h before the addition of the IgGs and that none of the three β_2GP-I antigens used in our *in vitro* experiments had intrinsic biologic activity, it is tempting to speculate that the IgG APS proinflammatory activity is exerted via constitutively bound β_2GPI perhaps synthesized by U937 cells. This would be in agreement with Conti et al. who showed β_2GPI mRNA expression by human monocytes and an abnormal β_2GPI cell surface expression on APS and SLE monocytes and high monocyte tissue factor expression [40].

In summary, carbohydrates chains are essential to cover cryptic epitopes present in the β_2GPI and diminish the recognition of soluble β_2GPI by anti-β_2GPI. The loss of sialic acid is sufficient to allow activation and differentiation of U937 cells by anti-β_2GPI–β_2GPI complexes.

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