Circulating Plasmablasts Contribute to Antiphospholipid Antibody Production, Associated with Type I Interferon Upregulation

Ryo Hisada[1], Masaru Kato[1], Eri Sugawara[1], Masatoshi Kanda[1], Yuichiro Fujieda[1], Kenji Oku[1], Toshiyuki Bohgaki[1], Olga Amengual[1], Tetsuya Horita[1], Shinsuke Yasuda[1] and Tatsuya Atsumi[1]

[1] Department of Rheumatology, Endocrinology and Nephrology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University

Correspondence to:

Dr. Masaru Kato

N15W7, Kita-Ku, 060-8638 Sapporo, Japan

Tel: +81-11-706-5915 Fax: +81-11-706-7710

ktmasaru@med.hokudai.ac.jp

Running head: Plasmablasts and aPL production

Summary

Background/Objective: Antiphospholipid antibodies (aPL) are pathogenic autoantibodies in antiphospholipid syndrome (APS). This study aimed to clarify the mechanism of aPL production.

Methods: T cell and B cell subsets were evaluated in peripheral blood mononuclear cells (PBMCs) of 26 primary APS (PAPS), 19 systemic lupus erythematosus-associated APS (SLE/APS) patients and 10 healthy controls. SLE or APS-related single nucleotide polymorphisms (SNP) were analysed in those patients. Interferon (IFN) score was calculated based on the mRNA expression of Ly6e, Mx1, IFIT1 and IFIT3 in PBMCs. PBMCs obtained from APS patients were cultured ex vivo following depletion of CD20 positive or negative B cells and the culture supernatants were applied to aPL measurements.

Results: In PAPS and SLE/APS patients, Th2, Th17 and plasmablasts were increased while regulatory T, memory B and regulatory B cells were decreased compared to healthy controls. Genetic analysis revealed that the increase of plasmablasts were more pronounced in patients carrying a risk allele of toll like receptor (TLR) 7 SNP rs3853839. IFN score was significantly higher in the risk allele carriers. Ex vivo experiments showed that aPL were present in the culture supernatant of PBMCs lacking CD20+CD19+ subset,

but not in that of cells lacking CD20-CD19+ subset.

Conclusions: Our data indicate an important role of plasmablasts in the production of aPL. Furthermore, the increase of plasmablasts was associated with TLR 7 and type I IFN, suggesting a common pathophysiology in SLE and APS. Targeting plasmablasts might be a novel, immunological therapeutic approach in the treatment of APS.

Essentials

- The mechanism of antiphospholipid antibodies (aPL) production remains unclear.
- We investigated lymphocyte subset, single nucleotide polymorphisms (SNP) and aPL-producing cells.
- The increase of circulating plasmablasts was associated with type I interferon upregulation.
- Our novel ex-vivo assay revealed circulating plasmablasts as a major source of aPL.

Introduction

Antiphospholipid syndrome (APS) is characterised by the occurrence of thrombosis and/or pregnancy morbidity associated with the persistent presence of antiphospholipid antibodies (aPL) [1, 2]. A number of clinical, ex vivo, in vitro and animal studies have supported aPL as pathogenic autoantibodies through inducing a procoagulant and proinflammatory state in APS and/or in systemic lupus erythematosus (SLE). For example, the antiphospholipid score, a quantitative marker calculated according to the titer and variety of aPL, is positively related to the risk of developing thrombosis [3]. In ex vivo and in vitro studies, circulating aPL have been shown to inhibit natural anticoagulants [4], suppress fibrinolytic activities [5], activate vascular endothelial cells [6], and promote the formation of neutrophil extracellular traps [7]. In a mouse model, the injection of patient-derived aPL potentiated thrombus formation [8]. In contrast to these functional findings, little is known about the mechanism of aPL production. Since aPL are not eliminated by immunosuppressive therapy including glucocorticoid, cyclophosphamide and rituximab [9], novel therapeutic approaches targeting aPL production are on currently unmet needs.

Immunophenotypic analyses of peripheral blood mononuclear cells (PBMCs)

have documented the specific lymphocyte maturation and differentiation in autoimmune diseases. A recent research demonstrated quantitative or functional defect of regulatory T (Treg) cells in a variety of autoimmune rheumatic disorders [10]. Circulating plasmablasts have been suggested to reflect immunological activity and to be an early biomarker in SLE [11]. The findings from these lymphocyte subset studies have not only contributed to our deep understanding of disease pathogenesis but also suggested novel cell-based therapeutic approaches.

Furthermore, the association between lymphocyte subsets and single nucleotide polymorphisms (SNP) have recently garnered attention. A study by Roederer et al [12] demonstrated the genetic architecture of the human immune system by pheno- and genotyping of 669 twins. Understanding how such genes interplay with the environment to determine immune protection and pathology is critical for unveiling the mechanisms of autoimmune diseases and future development of immunomodulatory therapies.

Nevertheless, so far, aPL-producing cells and the relationship of genetic background to lymphocyte subset in APS patients remains unclear. In this study, we evaluated lymphocyte subset analysis in APS patients and its association with SNP and mRNA gene expression. Then we established an ex vivo assay to evaluate cell's capability of producing aPL and investigated aPL-producing B cell subset by depleting specific

lymphocyte subset.

Methods

Patients and sample collection

Peripheral blood and serum samples were obtained from 45 APS patients; 26 primary APS (PAPS) patients (23 women and 3 men; mean age, 41.5 years) and 19 secondary APS patients associated with SLE (SLE/APS) (17 women and 2 men; mean age, 42.2 years) visiting Hokkaido University Hospital between January 2015 and December 2017. Clinical and laboratory diagnosis of SLE were based on the American College of Rheumatology 1997 criteria [13], while the Sydney-revised Sapporo criteria was utilized to diagnose APS [1]. At the sample collection, all patients with SLE were in clinical remission with SLE Disease Activity Index 2000 [14] of <4 and on low dose of glucocorticoids equivalent to 5 mg/day or less of prednisolone without any other immunosuppressive or antimalarial drugs. APS patients had maintained clinically quiescent state for over one year without active arterial or venous clots. In addition, 10 healthy controls (8 women and 2 men; mean age, 37.3 years) were enrolled as controls.

Baseline characteristics of PAPS, SLE/APS patients and healthy controls are shown in Table S1. They gave written informed consent for sample acquisition.

T and B cell subset analysis

PBMCs were isolated from peripheral blood using Ficoll-Paque Plus (GE Healthcare Biosciences). PBMCs were stained with specific monoclonal antibodies described in Table S2. Cells were differentiated into 20 subsets on a BD FACSAria™ II flow cytometer (BD bioscience) and analysed using the FlowJo software (Tree Star). The phenotype of T and B cell subsets was defined in Table S3 based on previous reports [15-18] with some modifications. Plasmablasts were defined as CD3- CD19+ CD20- CD27++ CD38++ cells according to the protocol by the Standardizing immunophenotyping for the Human Immunology Project [15].

Regulatory B cell subset analysis

Regulatory B (Breg) cells, defined by an interleukin (IL)-10 producing B-cell subset, were differentiated as described previously [19, 20]. PBMCs (2 x 10^6 cells) were

resuspended in 1 mL supplemented medium and cultured in the presence of 2 μL ODN 2006 Type B CpG (10 μg/ml, Miltenyi Biotec) for 66 h followed by restimulation for 6 h with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, Sigma-Aldrich), ionomycin (1 μg/ml, Sigma Aldrich), brefeldin A (1x solution, Biolegend). Stimulated cells were labelled with allophycocyanin (APC)-conjugated anti-CD19 (BioLegend) for 20 min in the dark. Next, cells were permeabilized with the FIX & PERM® Cell Permeabilization Kit (Invitrogen™) and labelled with phycoerythrin (PE)-conjugated anti-IL-10 for 20 min in the dark. After staining, cells were washed and immediately analysed.

Genetic analysis

Genomic DNAs from 14 female PAPS patients who did not use any immunosuppressive agents and gave informed consent were genotyped using predesigned Taqman® Sample-to-SNP™ Kit (Applied Biosystems) according to the manufacturer's instructions. These patients were included in the 26 PAPS patients enrolled for T and B cell subset analysis. Baseline characteristics of these patients are described in Table S4. Selected were 20 SNPs which have been shown to be associated with SLE or APS in previous studies (Table S5).

Analysis of TLR7 and type I IFN-regulated genes in PBMCs

The mRNA expression levels of toll like receptor (TLR) 7 were measured in PBMCs from 14 female PAPS patients. Total RNA was extracted from PBMCs using TRIzol (Invitrogen). RNA samples were reverse-transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen^{TM}). SYBR Green real-time PCR method was used to quantify the expression of TLR7 and four type I IFN–regulated genes including lymphocyte antigen 6 complex, locus E (LY6E), myxovirus resistance 1 (MX1), IFN-inducible protein with tetratricopeptide repeats (IFIT) 1 and IFIT3. The expression of GAPDH was used as an internal control. Primer pairs used in this study are shown in Table S6. IFN scores were calculated as described previously [21, 22].

Measurement of aPL in PBMCs culture supernatant

PBMCs were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (100 U/ml) in 96-wells flat bottom plates at a density of 5×10^4 cells/well with IL-6 (1 ng/ml, Biolegend), IL-21 (50 ng/ml, Biolegend), CD40 ligand (1 μg/ml, Biolegend) and a proliferation-inducing ligand

(APRIL) (300 ng/ml, PeproTech). Further, by cell sorting, CD20 positive B cells (CD19+CD20+ subset), CD20 negative B cells (CD19+CD20- subset) and PBMCs lacking CD20 positive B cells or CD20 negative B cells were generated and cultured under same conditions. Supernatants were collected at day 7 and applied to aPL measurements. Serum aPL profile of the patients for cell sorting analysis are shown in Table S7.

Anticardiolipin antibody detection assay

IgG and IgM of anticardiolipin antibodies (aCL) were assayed according to the standard enzyme linked immunosorbent assay (ELISA) [23]. Serum samples were tested at a 1:50 dilution, while culture supernatants were tested undiluted.

Anti-β2GPI/HLA class II complex antibody detection assay

Beta 2-glycoprotein I (β2GPI)/human leukocyte antigen (HLA) class II complex-overexpressing HEK293T cells were established to detect anti-β2GPI/HLA class II complex antibodies as previously reported [24]. HEK293T cells were transiently co-

transfected with plasmid vectors containing β2GPI, HLA-DR (HLA-DRA*01:01 and DRB1*07:01), which were kindly given from Prof. Arase, and green fluorescent protein (GFP) by polyethylenimine and were used for the assay two days after transfection. To detect anti-β2GPI/HLA class II complex antibodies in culture supernatants, 10 μL of the supernatant was added to 3×10^4 transfected HEK293T cells. After incubation on ice for 20 min, cells were washed with sterile phosphate buffered saline containing 1% bovine serum albumin and stained with APC-anti-human IgG or IgM antibodies for 10 min on ice. Specific IgG or IgM binding to the β2GPI/HLA-DR7 complex were calculated by subtracting mean fluorescence intensity (MFI) of IgG or IgM binding to GFP-negative cells from that to GFP-positive cells.

Statistical analysis

Statistical analysis was performed by Student's t-test, One-way ANOVA with post-hoc Tukey's test for normally distributed values, and Mann-Whitney U test, Dunn test after Kruskal Wallis test for values without Gaussian distribution. Correlation coefficients were assessed with the Spearman rank method. A paired t-test was used to compare samples under the same conditions. P values less than 0.05 were considered significant. All

statistical analyses were performed using GraphPad Prism 7 (Graphpad Software).

Results

The deviations of T and B cell subsets in APS patients

First, we performed lymphocyte subset analysis, which has so far been done for various autoimmune diseases and provided some key findings in their pathogenesis, in APS patients. The deviations of T and B cell subsets in these subjects are shown in Fig. 1 and Fig. S1. In T cell subset analysis, helper T (Th) cells, Th2 cells and Th17 cells were increased while total regulatory T cells, resting regulatory T cells and activated regulatory T cells were decreased in PAPS and SLE/APS patients compared to healthy controls (Fig. 1A). In B cell subset analysis, total memory B cells, pre-switched memory B cells and post-switched memory B cells were decreased (Fig. 1B) while plasmablasts were increased in PAPS and SLE/APS patients compared to healthy controls (Fig. 1C). Moreover, the decrease of IL-10 producing Breg cells were observed in PAPS and SLE/APS patients compared to healthy controls (Figs. 2A and B).

Conversely, there were no significant differences among three groups in the

proportion of total CD4+ T cells, Th1 cells, follicular helper T cells, central memory T cells, naïve T cells, effector memory T cells, effector T cells, transitional B cells, CD27-IgD- double negative B cells and naïve B cells (Fig. S1). The correlations between each aPL titer and plasmablast counts are shown in Fig. S2.

TLR 7 SNP rs3853839 was associated with the increase of plasmablasts and type I interferon upregulation.

Next, we investigated the association between disease-related SNPs and B cell subsets in APS patients. The prevalence of each listed allele among 14 PAPS patients is described in Table S8. As the results, the increase of plasmablasts and the decrease of memory B cells were associated with the SLE susceptible allele (GG) of TLR7 SNP rs3853839 (Figs. S3A and B, Table S9).

We also analysed the function of the TLR7 SNP rs3853839. Consistent with previous studies [22], IFN score, calculated using expression levels of four type I IFN-regulated genes including LY6E, MX1, IFIT1 and IFIT3, was significantly higher in subjects carrying the risk allele GG compared to those with GC allele ($p = 0.023$) (Fig. S3D), indicating a TLR7, type I IFN and B cell subset association. Difference in the

expression levels of TLR7 between GG and GC allele carriers did not reached to a statistical significance ($p = 0.059$) (Fig. S3C).

Circulating CD20 negative B cells as a major source of aPL

Given the increase of plasmablasts and its association with genetic variants in APS shown in the previous experiments, we hypothesized a particularly important role of plasmablasts in the APS pathogenesis and their capability of producing aPL. Prior to the analysis, PBMCs obtained from APS patients or healthy controls were ex vivo cultured in the presence of IL-6, IL-21, CD40 ligand and APRIL and culture supernatants were applied to aPL measurements. Anticardiolipin antibodies-IgG were detected in the culture supernatants of PBMCs from APS patients, particularly from the patients with a high titer of serum aCL-IgG, but not in those from healthy controls (Fig. 3A). Conversely, aCL-IgM were not detected in the culture supernatants of both patient and healthy PBMCs (Fig. 3B), suggesting either a relatively low sensitivity of ELISA for detecting aCL-IgM or a low capability of cells to produce aCL-IgM ex vivo. Next, PBMCs obtained from APS patients were applied to cell sorting and CD20 positive (CD19+CD20+ subset) or negative (CD19+CD20- subset) B cells were depleted (Fig. S4). These cells were cultured

in the same way as for the total PBMCs and culture supernatants were applied to aPL measurements. Anticardiolipin-IgG in the culture supernatants of PBMCs lacking CD20 positive B cells were detected less than in those of total PBMCs, while they were not detected in those of PBMCs lacking CD20 negative B cells (Fig. 3C). Since FACS-sorting does not significantly affect B cell viability (Fig. S5), these findings reveal CD20 negative B cells as a major source while CD20 positive B cells partially contributes to aCL-IgG production. To evaluate the role of non-B blood cells, we performed the culture of sorted B cells. Anticardiolipin-IgG were not detected both in the culture supernatants of CD20 positive B cells and in those of CD20 negative B cells (Fig. S6), supporting the contribution of non-B blood cells in aPL production.

Furthermore, the supernatants were also applied to the flow cytometric analysis of anti-β2GPI/HLA class II complex antibodies, another assay for detecting aPL [24]. Representative detection of serum anti-β2GPI/HLA class II complex -IgG and -IgM are presented in Figs. 4A and B. As in the detection of aCL-IgG, anti-β2GPI/HLA class II complex -IgM were detected in the culture supernatants of total PBMCs and PBMCs lacking CD20 positive B cells but not in those of PBMCs lacking CD20 negative B cells (Fig. 4D). Conversely, anti-β2GPI/HLA class II complex -IgG were not detected in the culture supernatants (Fig. 4C), again suggesting a difference in the assay sensitivity

between IgG and IgM detections.

Discussion

The pathophysiology of APS is incompletely understood. Most of the related studies have so far focused on aPL-cell interaction, intracellular signalling and subsequent expression of procoagulant genes such as tissue factor [25]. Given the pathogenicity of aPL, which is also supported by the fact that a subject with a high titer and/or a variety of aPL is on an increased risk of thrombosis [3], it would be of value to elucidate how and by which cells aPL are produced. Here, we have demonstrated a TLR7, type I IFN and B cell subset association. The increase of plasmablasts and the decrease of memory B cells observed in APS patients compared to healthy subjects were associated with the TLR7 SNP rs3853839. The SNP was further associated with the upregulation of type I IFN-regulated genes including LY6E, MX1, IFIT1 and IFIT3. Moreover, we established an ex vivo assay to evaluate cells' capability of producing aPL and showed CD20 negative B cells, which are composed mostly of plasmablasts, as a major source of aPL.

Interestingly, the deviations of T and B cell subsets of APS revealed in this study are similar to those of SLE with or without APS, which have been reported in previous

studies, including the increase of Th2, Th17 [26] and plasmablasts [11] and the decrease of Treg [27], memory B [28] and Breg cells [19]. These findings suggest a common pathophysiology in SLE and APS. In particular, plasmablasts may have a role in the development of autoimmune diseases by producing pathogenic cytokines and autoantibodies [29]. The role of Breg cells, a distinct B cell subset with immunomodulatory capacity by producing IL-10, IL-35 and transforming growth factor β, has recently been emerging [30]. The decrease of Breg cells is also found in other autoimmune rheumatic diseases such as antineutrophil cytoplasmic antibodies-associated vasculitis [31] and giant cell arteritis [32]. These T and B cell subset deviations are thought to be associated with pathogenesis and autoantibody production in APS.

Disease-related lymphocyte subset deviations were revealed by recent studies to be linked to genetic backgrounds. Our data for the first time demonstrated the association between B cell subset deviations and TLR7 gene in APS. Both B cell subset deviations, including plasmablast increase, and TLR7 SNP rs385389 have been reported to be associated with SLE. Moreover, TLR7 SNP rs385389 was shown by previous studies [22] as well as by ours to relate to upregulation of type I IFN, a central mediator in the development and pathogenesis of SLE. These findings again support a common pathophysiology in SLE and APS and could provide an explanation for some PAPS

patients developing SLE over the years. Furthermore, our data support the hypothesis that TLR7 expression induced by aPL [33] is associated with plasmablast differentiation via IFN signature. Since TLR7 gene is located on X chromosome, reactivation of inactivated X chromosome through epigenetic modifications could also contribute to upregulation of type I IFN in female SLE. Unlike CD40L gene which is also located on X chromosome and hypomethylated in SLE patients, TLR7 gene was shown to be resistant to demethylation [22]. Our genetic analysis included only female SLE patients, supporting a role of genetic backgrounds. The phenotypic differentiation of B cells to CD27-high populations including plasmablasts has been shown to be enhanced by plasmacytoid dendritic cells via their secretion of type I IFN upon agonizing TLR7 ligation [34], being consistent with a TLR7, type I IFN and B cell subset association demonstrated in this study.

In addition, B cells play both direct and indirect roles in aPL production. Indirect roles include cell-cell interactions and cytokine productions which may activate non-B blood cells, such as follicular helper T cells, and subsequently result in increased antibody production [35]. Compatible with this, our data suggest the indirect role of both CD20 positive and negative B cells in aPL production. Conversely, the direct role of CD20 negative B cells in aPL production is much clear than that of CD20 positive B cells. These

findings identified circulating CD20 negative B cells as dominant contributors in aPL production. Since only plasmablasts are CD20 negative populations among peripheral blood B cell compartments, including naïve, germinal centre and memory B cells [36], and bone marrow compartments including plasma cells are rarely found in the peripheral blood, our data strongly suggest a role of plasmablasts in the production of aPL. The anti-CD20 monoclonal antibody rituximab is currently used to treat some autoimmune diseases such as antineutrophil cytoplasmic antibodies-associated vasculitis and its therapeutic effect is partly through the reduction or disappearance of autoantibodies [37]. Our data are consistent with the results from a pilot phase II trial which could not observe the decrease of aPL titers following rituximab therapy [38] and suggest a need for novel therapeutic approaches targeting quantitative or functional abnormalities of plasmablasts.

This study has some limitations. First, some enrolled patients were treated with glucocorticoids, although the dose was equivalent to 5 mg/day or less of prednisolone, raising a possible influence on the results of lymphocyte subset. Second, limited number of patients in genetic analysis may miss the association of SNPs and lymphocyte subsets. Further analysis for larger number of the patients is needed to confirm our results. Third, there were not so many APS patients whose cells could be applied to our ex vivo assays to evaluate cells' capability of producing aPL, particularly following depletion of specific

subset, with sufficient sensitivity. Moreover, aCL-IgM and anti-β2GPI/HLA class II complex -IgG were not enough detected in ex vivo experiments possibly because of the relatively low sensitivity of their assay. Finally, the role of CD20 positive B cells and non-B blood cells in aPL production remains to be clarified by further studies since those cells might affect the interpretation of our data.

In conclusion, we revealed T and B cell subset deviations in APS and that the increase of plasmablasts was associated with TLR 7 and type I IFN, suggesting a common pathophysiology in SLE and APS. Ex vivo experiments showed that aPL-producing CD20 negative B cells circulate in peripheral blood of APS patients. This population, which is not targeted by anti-CD20 antibodies, reflects the active part of the aPL-specific B cell response and could represent a relevant target for therapeutic intervention in APS.

Addendum

R. Hisada designed, performed and analysed experiments and wrote the manuscript. M. Kato performed statistical analysis, interpreted the data, and drafted the manuscript. E. Sugawara, M. Kanda and Y. Fujieda supervised statistical analysis, interpreted the data, and revised the manuscript. K. Oku and T. Bohgaki interpreted the data and revised the

manuscript. O. Amengual and S. Yasuda helped in the design of the study and critically reviewed the manuscript. T. Atsumi discussed the data and revised the manuscript.

Acknowledgments

This study was supported in part by the Research Centre Network for Realization of Regenerative Medicine by the Japan Science and Technology Agency (JST) and the Japan Agency for Medical Research and Development (AMED) (project ID; 16ek0410022h0002). We thank H. Nakagawa for genetic analysis. We thank M. Shitamichi, Y. Kaneko and A. Yamada for excellent technical assistance.

Disclosure of Conflict of Interests

Dr. Atsumi reports personal fees from Chugai, during the conduct of the study; grants and personal fees from Astellas, grants and personal fees from Takeda, grants and personal fees from Mitsubishi Tanabe, grants and personal fees from Chugai, grants and personal fees from Pfizer, grants from Daiichi Sankyo, grants from Otsuka, personal fees from Eisai, personal fees from AbbVie, outside the submitted work. Dr. Yasuda reports grants

from Bristol-Myers Squibb Co., outside the submitted work. Dr. Kato reports grants from GSK, grants from Actelion, outside the submitted work. The other authors declare no conflict of interest associated with this manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Baseline characteristics of PAPS, SLE/APS and healthy control.

Table S2. Antibody panels for T and B cell subset analysis.

Table S3. Cell surface marker phenotype of T and B cell subset.

Table S4. Baseline characteristics of PAPS patients for genetic analysis.

Table S5. SNP list.

Table S6. Primer list.

Table S7. Serum aPL profile of the patients enrolled in the cell sorting analysis

Table S8. The prevalence of each listed allele among 14 PAPS patients.

Table S9. Association between disease-related SNPs and B cell subsets in patients who were not on immunosuppressive therapy

Fig. S1. Lymphocyte subset analysis in PAPS, SLE/APS and healthy controls.

Fig. S2. The correlations between each aPL titer and plasmablast counts.

Fig. S3. Association of plasmablasts and total memory B cells with TLR7 SNP rs3853839 in PAPS patients.

Fig. S4. FACS-sorted cells.

Fig. S5. B cell viability and total IgG production capability of unsorted and mock-sorted PBMCs.

Fig. S6. Ex-vivo aCL-IgG production in the culture supernatants of PBMC stimulated with IL-6 (1 ng/ml), IL-21 (50 ng/ml), CD40 ligand (1 μg/ml), APRIL (300 ng/ml).

References

1 Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH, PG DEG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis SA. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*. 2006; **4**: 295-306.

2 Amengual O, Atsumi T. Antiphospholipid syndrome, "the best prophet of the future". *Mod Rheumatol*. 2018: 1-8.

3 Otomo K, Atsumi T, Amengual O, Fujieda Y, Kato M, Oku K, Horita T, Yasuda S, Koike T. Efficacy of the antiphospholipid score for the diagnosis of antiphospholipid syndrome and its predictive value for thrombotic events. *Arthritis Rheum*. 2012; **64**: 504-12.

4 Urbanus RT, de Laat B. Antiphospholipid antibodies and the protein C pathway. *Lupus*. 2010; **19**: 394-9.

5 Bu C, Gao L, Xie W, Zhang J, He Y, Cai G, McCrae KR. beta2-glycoprotein i is a cofactor for tissue plasminogen activator-mediated plasminogen activation. *Arthritis Rheum*. 2009; **60**: 559-68.

6 Zhang J, McCrae KR. Annexin A2 mediates endothelial cell activation by

antiphospholipid/anti-beta2 glycoprotein I antibodies. *Blood*. 2005; **105**: 1964-9.

7 Yalavarthi S, Gould TJ, Rao AN, Mazza LF, Morris AE, Nunez-Alvarez C, Hernandez-Ramirez D, Bockenstedt PL, Liaw PC, Cabral AR, Knight JS. Release of neutrophil extracellular traps by neutrophils stimulated with antiphospholipid antibodies: a newly identified mechanism of thrombosis in the antiphospholipid syndrome. *Arthritis Rheumatol*. 2015; **67**: 2990-3003.

8 Arad A, Proulle V, Furie RA, Furie BC, Furie B. beta(2)-Glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. *Blood*. 2011; **117**: 3453-9.

9 Cervera R. Antiphospholipid syndrome. *Thromb Res*. 2017; **151 Suppl 1**: S43-S7.

10 Bernard F, Romano A, Granel B. [Regulatory T cells and systemic autoimmune diseases: systemic lupus erythematosus, rheumatoid arthritis, primary Sjogren's syndrome]. *Rev Med Interne*. 2010; **31**: 116-27.

11 Jacobi AM, Mei H, Hoyer BF, Mumtaz IM, Thiele K, Radbruch A, Burmester GR, Hiepe F, Dorner T. HLA-DRhigh/CD27high plasmablasts indicate active disease in patients with systemic lupus erythematosus. *Ann Rheum Dis*. 2010; **69**: 305-8.

12 Roederer M, Quaye L, Mangino M, Beddall MH, Mahnke Y, Chattopadhyay P, Tosi

I, Napolitano L, Terranova Barberio M, Menni C, Villanova F, Di Meglio P, Spector TD, Nestle FO. The genetic architecture of the human immune system: a bioresource for autoimmunity and disease pathogenesis. *Cell*. 2015; **161**: 387-403.

13 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum*. 1997; **40**: 1725.

14 Gladman DD, Ibanez D, Urowitz MB. Systemic lupus erythematosus disease activity index 2000. *The Journal of rheumatology*. 2002; **29**: 288-91.

15 Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the Human Immunology Project. *Nat Rev Immunol*. 2012; **12**: 191-200.

16 Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, Foucat E, Dullaers M, Oh S, Sabzghabaei N, Lavecchio EM, Punaro M, Pascual V, Banchereau J, Ueno H. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011; **34**: 108-21.

17 Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochov G, Sakaguchi S. Functional delineation and differentiation

dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009; **30**: 899-911.

18 Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. 2006; **203**: 1701-11.

19 Heinemann K, Wilde B, Hoerning A, Tebbe B, Kribben A, Witzke O, Dolff S. Decreased IL-10(+) regulatory B cells (Bregs) in lupus nephritis patients. *Scand J Rheumatol*. 2016; **45**: 312-6.

20 Matsushita T, Hamaguchi Y, Hasegawa M, Takehara K, Fujimoto M. Decreased levels of regulatory B cells in patients with systemic sclerosis: association with autoantibody production and disease activity. *Rheumatology (Oxford)*. 2016; **55**: 263-7.

21 Feng X, Wu H, Grossman JM, Hanvivadhanakul P, FitzGerald JD, Park GS, Dong X, Chen W, Kim MH, Weng HH, Furst DE, Gorn A, McMahon M, Taylor M, Brahn E, Hahn BH, Tsao BP. Association of increased interferon-inducible gene expression with disease activity and lupus nephritis in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2006; **54**: 2951-62.

22 Shen N, Fu Q, Deng Y, Qian X, Zhao J, Kaufman KM, Wu YL, Yu CY, Tang Y, Chen JY, Yang W, Wong M, Kawasaki A, Tsuchiya N, Sumida T, Kawaguchi Y, Howe HS, Mok MY, Bang SY, Liu FL, et al. Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus. *Proc Natl Acad Sci U S A*. 2010; **107**: 15838-43.

23 Harris EN, Gharavi AE, Patel SP, Hughes GR. Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986. *Clin Exp Immunol*. 1987; **68**: 215-22.

24 Tanimura K, Jin H, Suenaga T, Morikami S, Arase N, Kishida K, Hirayasu K, Kohyama M, Ebina Y, Yasuda S, Horita T, Takasugi K, Ohmura K, Yamamoto K, Katayama I, Sasazuki T, Lanier LL, Atsumi T, Yamada H, Arase H. beta2-Glycoprotein I/HLA class II complexes are novel autoantigens in antiphospholipid syndrome. *Blood*. 2015; **125**: 2835-44.

25 Chaturvedi S, McCrae KR. Diagnosis and management of the antiphospholipid syndrome. *Blood Rev*. 2017; **31**: 406-17.

26 Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Th1/Th2/Th17/Treg cytokine imbalance in systemic lupus erythematosus (SLE) patients: Correlation with disease activity. *Cytokine*. 2015; **72**: 146-53.

27 Ohl K, Tenbrock K. Regulatory T cells in systemic lupus erythematosus. *Eur J Immunol*. 2015; **45**: 344-55.

28 Rodriguez-Bayona B, Ramos-Amaya A, Perez-Venegas JJ, Rodriguez C, Brieva JA. Decreased frequency and activated phenotype of blood CD27 IgD IgM B lymphocytes is a permanent abnormality in systemic lupus erythematosus patients. *Arthritis Res Ther*. 2010; **12**: R108.

29 Tiburzy B, Kulkarni U, Hauser AE, Abram M, Manz RA. Plasma cells in immunopathology: concepts and therapeutic strategies. *Semin Immunopathol*. 2014; **36**: 277-88.

30 Menon M, Blair PA, Isenberg DA, Mauri C. A Regulatory Feedback between Plasmacytoid Dendritic Cells and Regulatory B Cells Is Aberrant in Systemic Lupus Erythematosus. *Immunity*. 2016; **44**: 683-97.

31 Wilde B, Thewissen M, Damoiseaux J, Knippenberg S, Hilhorst M, van Paassen P, Witzke O, Cohen Tervaert JW. Regulatory B cells in ANCA-associated vasculitis. *Ann Rheum Dis*. 2013; **72**: 1416-9.

32 van der Geest KS, Abdulahad WH, Chalan P, Rutgers A, Horst G, Huitema MG, Roffel MP, Roozendaal C, Kluin PM, Bos NA, Boots AM, Brouwer E. Disturbed B cell homeostasis in newly diagnosed giant cell arteritis and polymyalgia rheumatica.

Arthritis Rheumatol. 2014; **66**: 1927-38.

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

34 Gujer C, Sundling C, Seder RA, Karlsson Hedestam GB, Lore K. Human and rhesus plasmacytoid dendritic cell and B-cell responses to Toll-like receptor stimulation. *Immunology*. 2011; **134**: 257-69.

35 Hoffman W, Lakkis FG, Chalasani G. B Cells, Antibodies, and More. *Clin J Am Soc Nephrol*. 2016; **11**: 137-54.

36 Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. *Nat Rev Immunol*. 2006; **6**: 394-403.

37 Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, Savage CO, Segelmark M, Tesar V, van Paassen P, Walsh D, Walsh M, Westman K, Jayne DR, European Vasculitis Study G. Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. *N Engl J Med*. 2010; **363**: 211-20.

38 Erkan D, Vega J, Ramon G, Kozora E, Lockshin MD. A pilot open-label phase II trial of rituximab for non-criteria manifestations of antiphospholipid syndrome.

Arthritis Rheum. 2013; **65**: 464-71.

Figure legends

Fig. 1. Lymphocyte subset analysis in PAPS, SLE/APS and healthy controls. (A) The percentage of helper T cells, Th2 cells and Th17 cells, total Treg cells, resting Treg cells and activated Treg cells per CD4+ T cells. (B) The percentage of total memory B cells, pre-switched memory B cells and post-switched memory B cells per CD19+ B cells. (C) The percentage of plasmablasts per CD19+ B cells. (D) Representative dot plots depicting plasmablasts. *$p<0.05$. Th, helper T; Treg, regulatory T; PAPS, primary antiphospholipid syndrome; SLE/APS, systemic lupus erythematosus-associated antiphospholipid syndrome.

Fig. 2. Breg cell subset analysis in PAPS, SLE/APS and healthy controls. (A) The percentages of Breg cells per CD19+ B cells. (B) Representative dot plots depicting IL-10+ B cells following stimulation. *$p<0.05$. Breg, regulatory B; PAPS, primary antiphospholipid syndrome.

Fig. 3. Ex-vivo aCL-IgG/IgM production in the culture supernatants of PBMCs stimulated with IL-6 (1 ng/ml), IL-21 (50 ng/ml), CD40 ligand (1 μg/ml), APRIL (300 ng/ml). (A) aCL-IgG and (B) -IgM detected by ELISA in the culture supernatants of

PBMCs from APS patients or healthy controls. (C) aCL-IgG detected by ELISA in the culture supernatants of total PBMCs, PBMCs depleted of CD20+ B cells or PBMCs depleted of CD20- B cells from four patients. Each point represents one individual patient or healthy control. *p<0.05. aCL, anticardiolipin antibodies; GPL-U, G phospholipids units; MPL-U, M phospholipids units; PBMCs, peripheral blood mononuclear cells.

Fig. 4. Ex-vivo aβ2GPI/HLA class II complexes -IgG/IgM production in the culture supernatants of PBMCs stimulated with IL-6, IL-21, CD40 ligand, APRIL.

(A) aβ2GPI/HLA class II complexes -IgG and (B) -IgM detected by cell-based assay using β2GPI, HLA-DR7 and GFP overexpressing HEK293T cells in the serum from APS patients or healthy controls. (C) aβ2GPI/HLA class II complexes -IgG and (D) -IgM in culture supernatants of total PBMCs, PBMCs depleted of CD20+ B cells or PBMCs depleted of CD20- B cells detected by cell-based assay from three APS patients. Specific IgG/IgM binding to the β2GPI/HLA-DR7 complexes were calculated by subtracting the MFI of IgG/IgM binding to GFP-negative cells (shaded histogram) from the MFI of IgG/IgM binding to GFP-positive cells (open histogram) by flow cytometry. Data from representative of at least 3 independent experiments. *p<0.05. β2GPI, anti beta 2-

glycoprotein I; HLA, human leukocyte antigen; PBMCs, peripheral blood mononuclear cells; MFI, mean fluorescence intensity.

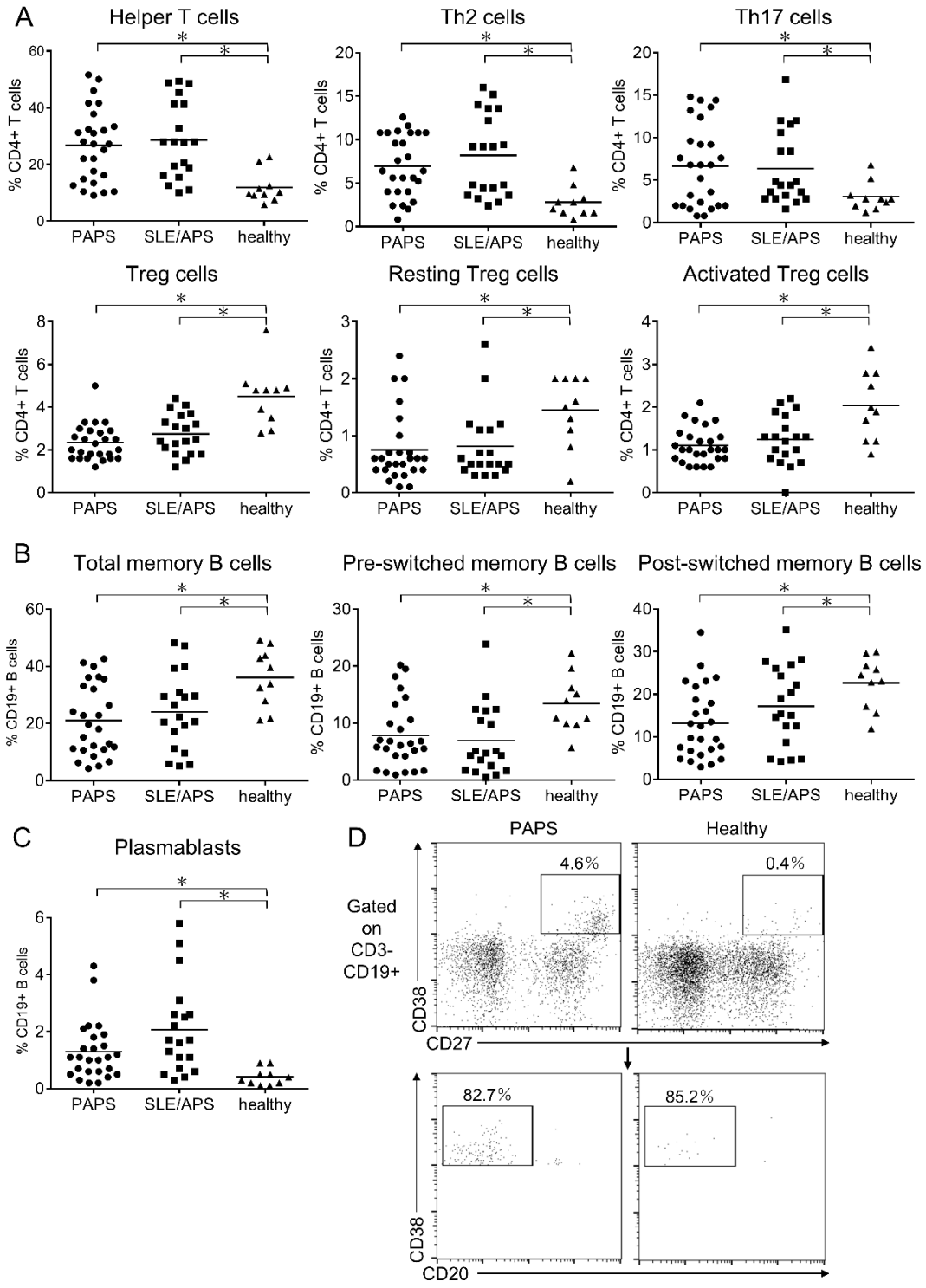

Fig. 1. Lymphocyte subset analysis in PAPS, SLE/APS and healthy controls. (A) The

percentage of helper T cells, Th2 cells and Th17 cells, total Treg cells, resting Treg cells and activated Treg cells per CD4+ T cells. (B) The percentage of total memory B cells, pre-switched memory B cells and post-switched memory B cells per CD19+ B cells. (C) The percentage of plasmablasts per CD19+ B cells. (D) Representative dot plots depicting plasmablasts. *p<0.05. Th, helper T; Treg, regulatory T; PAPS, primary antiphospholipid syndrome; SLE/APS, systemic lupus erythematosus-associated antiphospholipid syndrome.

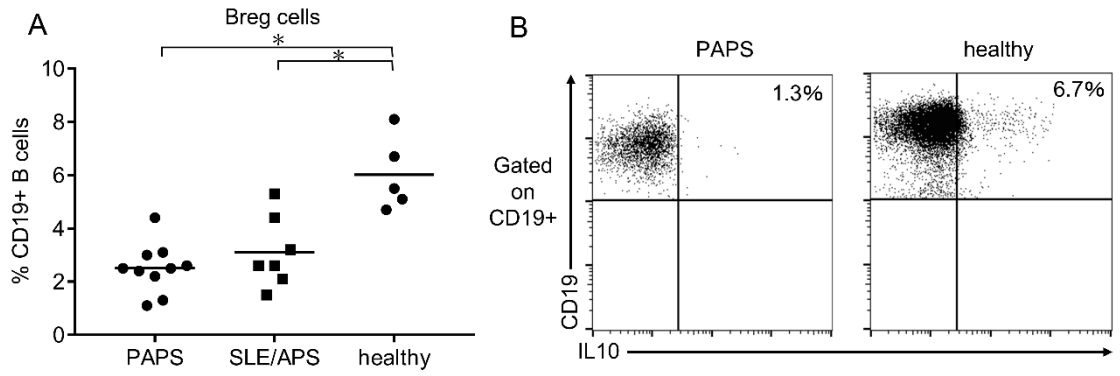

Fig. 2. Breg cell subset analysis in PAPS, SLE/APS and healthy controls. (A) The percentages of Breg cells per CD19+ B cells. (B) Representative dot plots depicting IL-10+ B cells following stimulation. *p<0.05. Breg, regulatory B; PAPS, primary antiphospholipid syndrome.

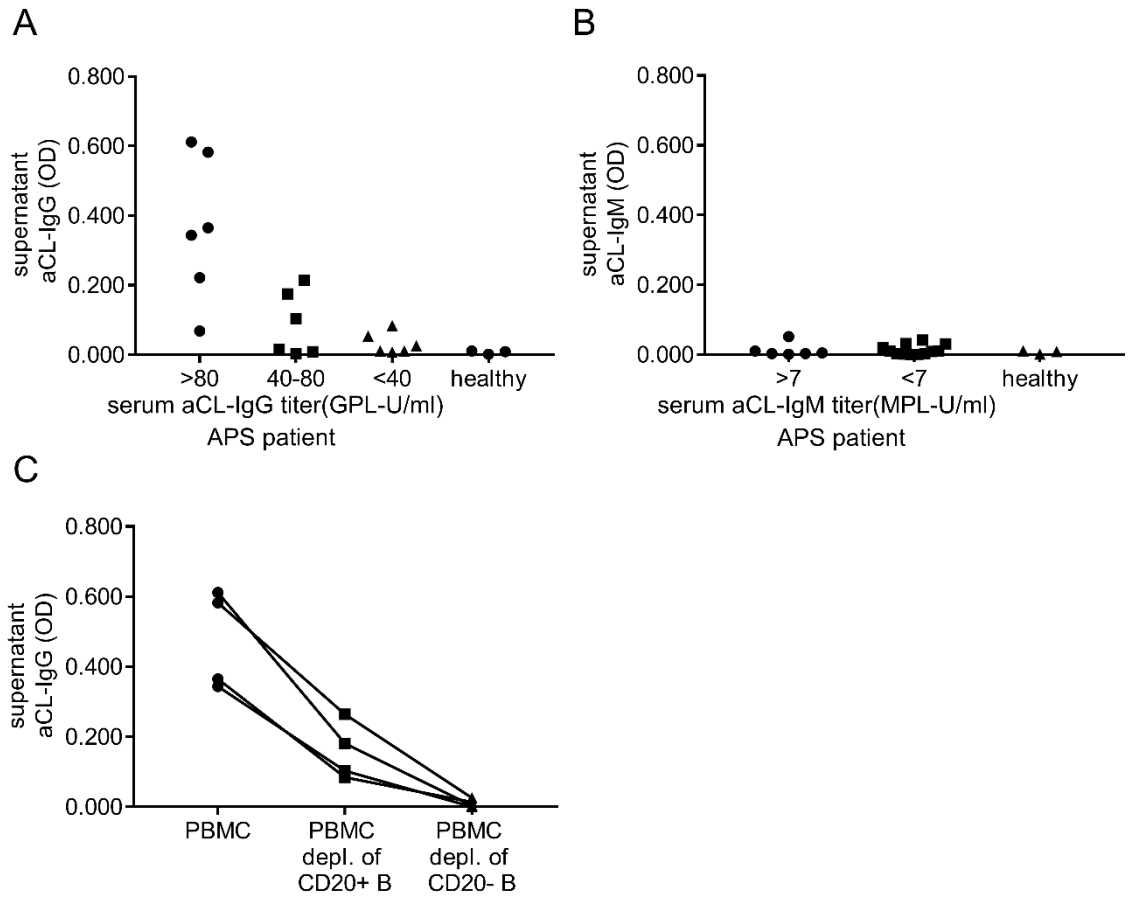

Fig. 3. Ex-vivo aCL-IgG/IgM production in the culture supernatants of PBMCs stimulated with IL-6 (1 ng/ml), IL-21 (50 ng/ml), CD40 ligand (1 μg/ml), APRIL (300 ng/ml). (A) aCL-IgG and (B) -IgM detected by ELISA in the culture supernatants of PBMCs from APS patients or healthy controls. (C) aCL-IgG detected by ELISA in the culture supernatants of total PBMCs, PBMCs depleted of CD20+ B cells or PBMCs depleted of CD20- B cells from four patients. Each point represents one individual patient or healthy control. *$p<0.05$. aCL, anticardiolipin antibodies; GPL-U, G phospholipids units; MPL-U, M phospholipids units; PBMCs, peripheral blood mononuclear cells.

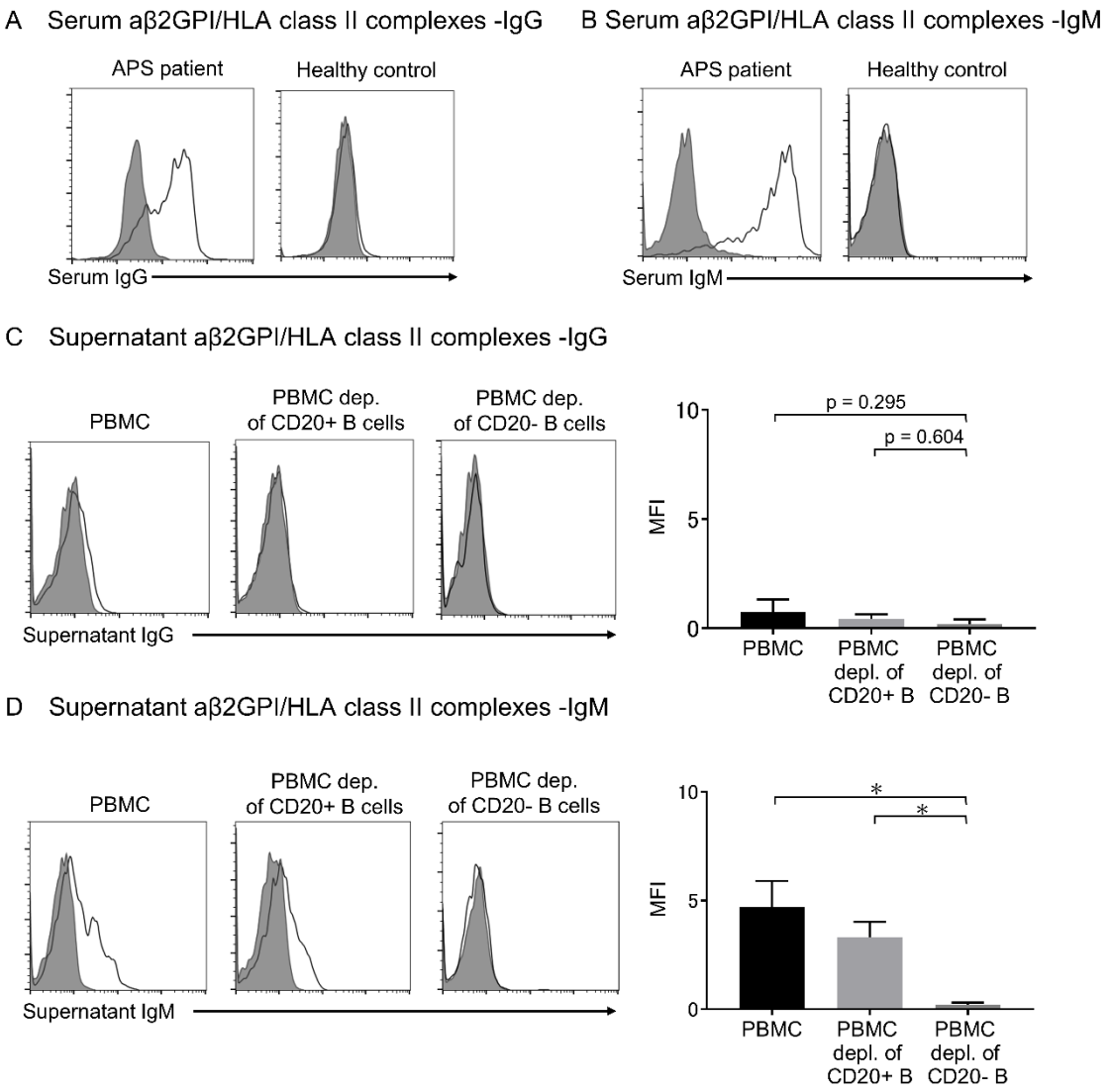

Fig. 4. Ex-vivo aβ2GPI/HLA class II complexes -IgG/IgM production in the culture supernatants of PBMCs stimulated with IL-6, IL-21, CD40 ligand, APRIL.

(A) aβ2GPI/HLA class II complexes -IgG and (B) -IgM detected by cell-based assay using β2GPI, HLA-DR7 and GFP overexpressing HEK293T cells in the serum from APS

patients or healthy controls. (C) aβ2GPI/HLA class II complexes -IgG and (D) -IgM in culture supernatants of total PBMCs, PBMCs depleted of CD20+ B cells or PBMCs depleted of CD20- B cells detected by cell-based assay from three APS patients. Specific IgG/IgM binding to the β2GPI/HLA-DR7 complexes were calculated by subtracting the MFI of IgG/IgM binding to GFP-negative cells (shaded histogram) from the MFI of IgG/IgM binding to GFP-positive cells (open histogram) by flow cytometry. Data from representative of at least 3 independent experiments. *$p<0.05$. β2GPI, anti beta 2-glycoprotein I; HLA, human leukocyte antigen; PBMCs, peripheral blood mononuclear cells; MFI, mean fluorescence intensity.