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Memory B cell pool of autoimmune pulmonary alveolar proteinosis patients contains higher frequency of GM-CSF autoreactive B cells than healthy subjects*

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ABSTRACT

The IgG-type neutralizing GM-CSF autoantibody (GMAb) is known to be the causative agent for autoimmune pulmonary alveolar proteinosis (APAP). Previous studies report that serum levels of IgG-GMAb are approximately 50-fold higher in APAP patients than in healthy subjects (HS). Serum levels of IgM-GMAb are also higher in APAP patients than in HS, but this has been assumed to be an etiological bystander. However, the mechanism for the excessive production of IgG-GMAb in APAP remains unclear. To investigate this, we detected putative GMAb-producing B cells (PGMPB) by inoculated B cells from the peripheral blood of APAP patients, HS, and umbilical cord blood mononuclear cells (UCBMNs) with Epstein-Barr virus. Both ELISA and ELISPOT assays showed that IgM-type GMAb was consistently and frequently present in all three groups, whereas IgG-type GMAb was high only in APAP patients, in whom it was exclusively produced in memory B cells and not in naive B cells. Since PGMPB in UCBMNs produced IgM-GMAb, but not IgG-GMAb, to the same extent as in HS and APAP patients, most IgM-GMAb reacted with GM-CSF in a non-specific manner. The memory B cell pool of APAP patients contain higher frequency of PGMPB than that of healthy subjects.

1. Introduction

Pulmonary alveolar proteinosis (PAP) is a rare lung disease characterized by the excessive accumulation of surfactant in the alveoli and the terminal bronchioles [1]. Autoimmune PAP (APAP) is the primary variant of PAP; it accounts for 91% of the disease and has an incidence of 1.65 cases per million in the general population of Japan [2]. Patients with APAP harbor a high level of autoantibodies against granulocyte/macrophage-colony-stimulating factor (GM-CSF) in their serum as well as in their lungs [3,4]. GM-CSF autoantibodies (GMAb) neutralize the biological activity of GM-CSF [5], impairing alveolar macrophage (AM)-mediated pulmonary surfactant clearance. GMAb purified from a patient reproduced PAP after its transfer into nonhuman primates, indicating that GMAb directly causes PAP [6].

Cytokine autoantibodies have been reported in various diseases, and are increasingly being recognized as potential contributors to acquired immune deficiency, immune dysregulation, and autoimmunity [7,8]. Although causality has not been established in all cases, these autoantibodies are generally polyclonal IgG in nature and may mediate diverse infectious and/or immunological manifestations, depending on the targeting cytokine. Cytokine autoantibodies are also known to occur in the sera of normal subjects, although at lower levels. According to a study by Svenson et al. [9], GMAb was the most common cytokine autoantibody found in pharmaceutically prepared intravenous immunoglobulin (IVIG). GMAbs were ubiquitously and consistently detected in all healthy subjects at low levels [10], sufficient to rheostatically regulate multiple myeloid functions. They exist in IgG, IgM, and IgA isotypes, but the IgG isotype accounts for more than 90% of antibodies of all types [11]. The binding avidity of the IgM isotype is 100-fold lower than that of the IgG isotype, and its neutralizing capacity is

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extremely weak, with an IC_{50} that is 20,000-fold higher than that of IgG-GMAb [11]. There is insufficient information on IgA-GMAb in peripheral blood, and thus its pathogenic role remains unclear. IgG-GMAb is therefore clearly pathogenic, whereas IgM-GMAb is thought to be an etiological bystander [11].

Transforming human B cells with Epstein-Barr virus (EBV) is a useful method for producing fully human monoclonal antibodies (mAb) [12]. EBV is a member of the B-lymphotropic gamma herpes virus family that infects B cells [13]. EBV infection leads to the proliferation and transformation of B cells into immortalized lymphoblastoid cells (LCL) [14]. This unique ability of EBV is extensively used to generate immortalized B cells that can produce antigen-specific human mAbs [15]. However, the EBV life cycle and transformation produces different results in different types of B cells. Transformed B cells from the umbilical cord and maternal blood produce exclusively IgM-type GMAb [16].

To conduct this study, we produced circulating autoreactive B cells against GM-CSF (putative GMAb-producing B cells: PGMPB) that produced GMAb by stimulating B cells with EBV or other B cell mitogens *in vitro*. PGMPB that have been detected in peripheral blood samples from APAP patients are thought to harbor surface GMAb for B cell receptors [17], and are thus plausible precursor cells for GMAb-producing plasma cells. However, the frequencies and antibody isotypes of PGMPB remain unclear. To elucidate this, we evaluated the frequencies of PGMPB in the peripheral blood of APAP or HS, and in UCBMNs after inoculating with EBV.

2. Materials and methods

2.1. Subjects

Eleven APAP patients (two females and nine males, aged between 15 and 61 years)(Table 1) and ten healthy subjects (HS) were enrolled in this study. We also obtained UCBMNs samples from three normal full-term newborn infants. APAP was diagnosed using high-resolution computed tomography (HRCT) findings, lung biopsy, or bronchoalveolar lavage findings. The diagnosis was confirmed by measuring the concentration of serum GMAb according to the diagnostic criteria (http://www.pap-guide.jp/en/). All patients showed mild to severe respiratory failure, with a mean PaO_2 of 70.4 ± 6.7 Torr, and were categorized according to disease severity score (DSS) at enrollment, as previously described [18]. All samples were collected at the Niigata University Medical and Dental Hospital. Written informed consent was obtained from all individual participants and/or their legal guardians, including five pregnant women, at the beginning of this study. For HS obtained from under the age of 18 years, all informed consents were obtained from each parents. This study was conducted according to the principles of the Declaration of Helsinki, and the Institutional Ethics Review Committee of Niigata University approved the study including study's protocol on December 1, 2008 (No. 796).

Table 1
Demographics data of whole enrolled patients.

patient	age	gender	respiratory symptom	DSS**	$PaCO_2$(*Torr*)	PaO_2(*Torr*)
patient 1	28	M	DOE	2	38	74
patient 2	22	M	DOE	4	42	59
patient 3	55	F	DOE	3	43	68
patient 4	57	M	DOE, sputum	3	41	67
patient 5	48	F	none	1	39	76
patient 6	61	M	DOE, cough	3	35	66
patient 7	31	M	none	3	39	64
patient 8	58	F	DOE, sputum	3	31	73
patient 9	38	M	cough	2	41	79
patient 10	44	M	DOE	3	37	67
patient 11	15	M	DOE	2	38	81

Definition of abbreviations: $AaDO_2$; alveolar–arterial oxygen difference; DOE; dyspnea on effort, DSS; disease severarity score, GMAb; anti granulocyte/macrophage-colony stimulating factor autoantibody, $PaCO_2$; partial pressure of arterial CO_2 measured in arterial blood, PaO_2; partial pressure of arterial oxygen measured in arterial blood. *Patients with aPAP were categorized by disease severity score at enrollment, as previously described reference number.

2.2. Reagents

The B95-8 cell line that was used to generate the EBV was maintained according to a previous report [16]. In brief, the cell line (American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640 complete medium supplemented with 10% fetal bovine serum (FBS). EBV was collected from the two-day culture supernatants of B95-8 cells seeded at a concentration of 3.7×10^4 cells/mL. Centrifuged supernatants were filtered three times (0.45 μm filters) and stored at −80 °C. Monoclonal IgG-GMAb was kindly provided by Dr Kenzo Takada (Evec, Sapporo, Japan) and was used as the standard for the ELISA. The IgM-GMAb standard was prepared as described previously [6]. Briefly, IgG was removed from pooled sera from APAP patients and loaded onto GM-CSF-coupled NHS HiTrap columns and eluted with glycine-HCl (10 mM, pH 2.8). The IgM/A-GMAb fraction was further purified by loading it onto the HiTrap IgM Purification HP (GE Healthcare) columns, according to the manufacturer's instructions. The purity of isolated GMAbs was close to 100% for IgM, as determined by ELISA (Bethyl Laboratories, Montgomery, TX). The following mAbs were used in this study: Alexa-Fluor 488-conjugated anti-CD38 and phycoerythrin (PE)-conjugated anti-CD27 were purchased from BD Bioscience (San Diego, CA); PE-Texas Red (ECD)-conjugated CD19 was purchased from Beckman Coulter; and PE-cyanine 5 (PE-Cy5)-conjugated anti-CD138 and CD24 were purchased from Beckman Coulter (Brea, CA).

2.3. Isolation of PBMCs from peripheral blood and cord blood

PBMCs were isolated from 20 mL of heparinized blood by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare UK, Buckinghamshire, UK).

2.4. In vitro production of GMAb from PBMCs by B cell stimulation

PBMCs were suspended in 1.0 mL of RPMI 1640 with 10% FBS at a concentration of 2.0×10^6 cells/mL and inoculated with either 1 mL of the EBV supernatant, 10 μg/ml pokeweed mitogen (PWM, Sigma) [19], 10 μg/mL of oligo-CpG (5′-tcgtcgttttgtcgttttgtcgtt-3′, Sigma), 1 ng/mL of phorbol myristate acetate (PMA; Sigma), or 10 μg/mL of lipopolysaccharide (LPS, derived from *Escherichia coli*, Sigma). These suspensions were incubated at 37 °C under 5% CO_2. Aliquots of the culture supernatant were collected every week for five weeks and used for measurement of GMAb concentration.

2.5. Measurement of GMAb concentration

The concentration of GMAb was measured using an ELISA described in a previous report [11]. In brief, IgG- and IgM-isotype GMAb in the culture supernatant was directly captured on *Saccharomyces*-derived recombinant human (rh) GM-CSF (Leukine, Genzyme, MA) and detected using peroxidase-labeled murine Fcμ and Fcγ antibody, respectively. The human monoclonal antibody described above and purified IgM-polyclonal antibody [11] were used as standards for the IgG and IgM isotypes, respectively. The lower detection limit was defined as the concentration corresponding to two standard deviations (2SD) of the blank optical density; this was set at 0.78 and 1.25 ng/mL for IgG- and IgM-GMAb, respectively. The concentrations under these values were defined as 0 ng/ml.

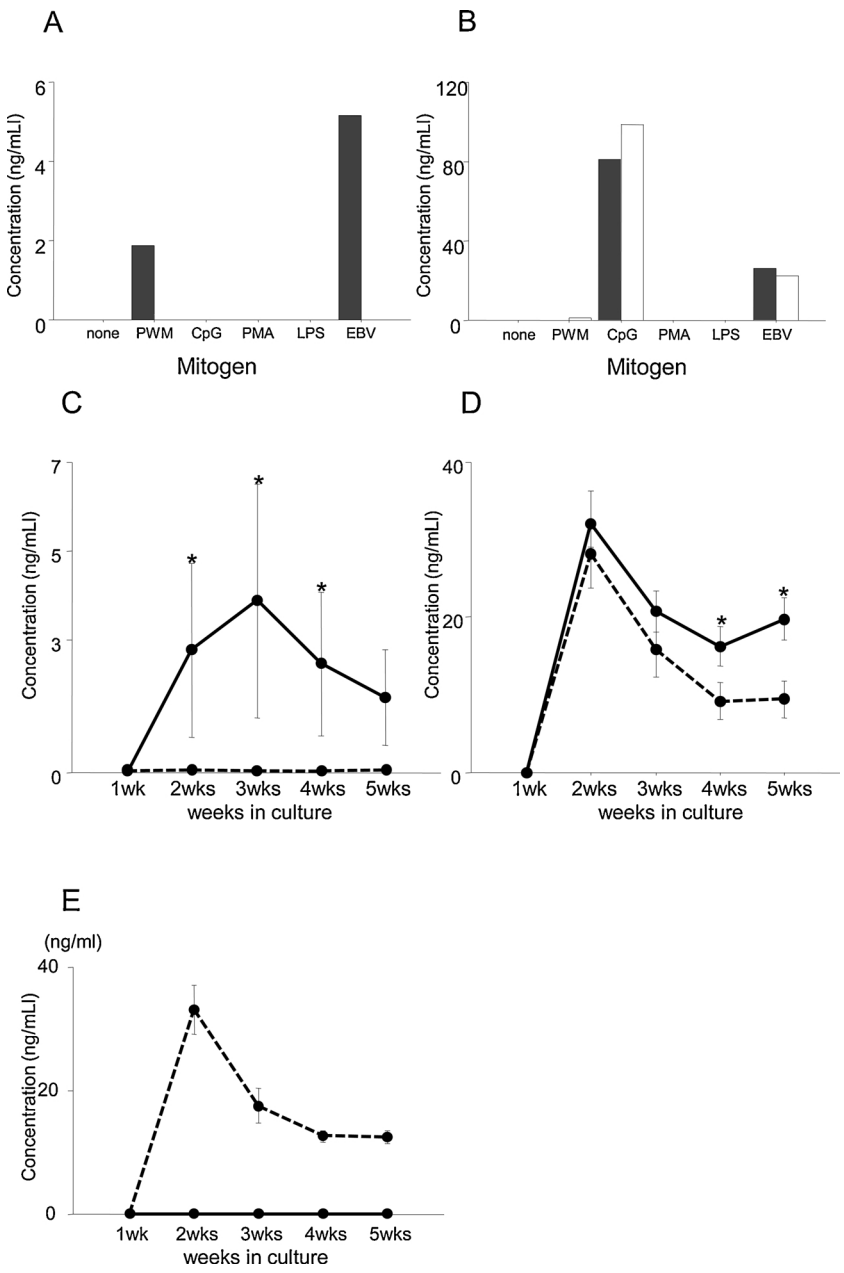

Fig. 1. The concentration of isotypes of the GM-CSF autoantibody derived from three types of blood sample, co-cultured with various mitogens or for various lengths of time. (A, B) PBMCs were co-cultured with the following mitogens: pokeweed mitogen (PWM, 10 μg/mL), oligo-CpG (CpG, 5′-tcgtcgttttgtcgttttgtcgtt-3′, 5 μg/mL), phorbol myristate acetate (PMA, 1 ng/mL), lipopolysaccharide (LPS, derived from *Escherichia coli*, 10 μg/mL), and Epstein-Barr virus-containing cultured media, B95-8 strain (EBV). The concentration of IgG-GMAb (A) and IgM-GMAb (B) in cultured media from APAP patients (gray) or HS (white) were determined as described in the Materials and Methods. Data are for a representative case. (C, D) PBMCs were co-cultured with the supernatant from the B95-8 cell line for five weeks. IgG-GMAb (C) or IgM-GMAb (D) was derived from the media of PBMCs from APAP patients (solid line, n = 10) or HS (dashed line, n = 10). Statistical difference of IgG-GMAb concentration was observed at 2, 3 and 4 weeks, and IgM-GMAb was 4 and 5 weeks. (E) GMAb production from EBV-infected UCBMNs. Concentration of IgG-(solid line) or IgM-GMAb (dashed line) from umbilical cord blood mononuclear cells (UCBMNCs, n = 3).

2.6. ELISPOT assay for detecting GMAb-producing B cells

The ELISPOT assay was performed as described previously, with a minor modification [20,21]. Briefly, 96-well microtiter plates with a nitrocellulose bottom (Millipore, Bedford, MA, US) that had been previously hydrophilized with 35% ethanol were coated with 2.0 μg/mL of *Saccharomyces*-derived rhGM-CSF in sterile phosphate buffer saline (PBS)/0.1% bovine serum albumin (BSA) overnight at 4 °C. Unbound rhGM-CSF was removed by washing twice with sterile PBS, and wells were blocked with sterile PBS/1% BSA for 6 h at 4 °C. Subsequently, $1.0–2.0 \times 10^6$ of either PBMCs or sorted cells were suspended in 1–2 mL of EBV B95-8 supernatant. Aliquots were transferred to the 96-well plate, which was incubated for 14 d at 37 °C under 5% CO_2. After the wells had been washed with distilled water and PBS, each well was blocked with PBS/1% BSA/0.05% Tween-20 (PBST) for 3 h. After washing with PBST three times, 1 μg/mL of alkaline phosphatase-conjugated murine anti-human Fcμ or Fcγ mAb was added to each well, and the plate was incubated for 30 min at room temperature. After washing the plate with PBST three times, the 3-amino-9-ethylcarbazole (ACE) substrate solution (BD) was added. The plate was kept in the dark for 30 min to develop the color reaction, and the reaction was stopped by removing the substrate solution. The number of spots was counted manually under a dissecting microscope.

2.7. Cell sorting of B cell fractions

B cells were isolated using a Pan B cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry confirmed the purity of isolated B cells. To further separate them into naive and memory B cell fractions, the cells were incubated with MACS antibodies against CD27 (Miltenyi Biotec). The fraction sorted by the MACS magnetic sorting system was used as memory B cells, and the flow-through fraction was designated as naive B cells.

2.8. Flow cytometry analysis of B cells

The isolation of the memory and naive B cell fractions from the PBMC was evaluated using flow cytometry analysis. Briefly, cells were

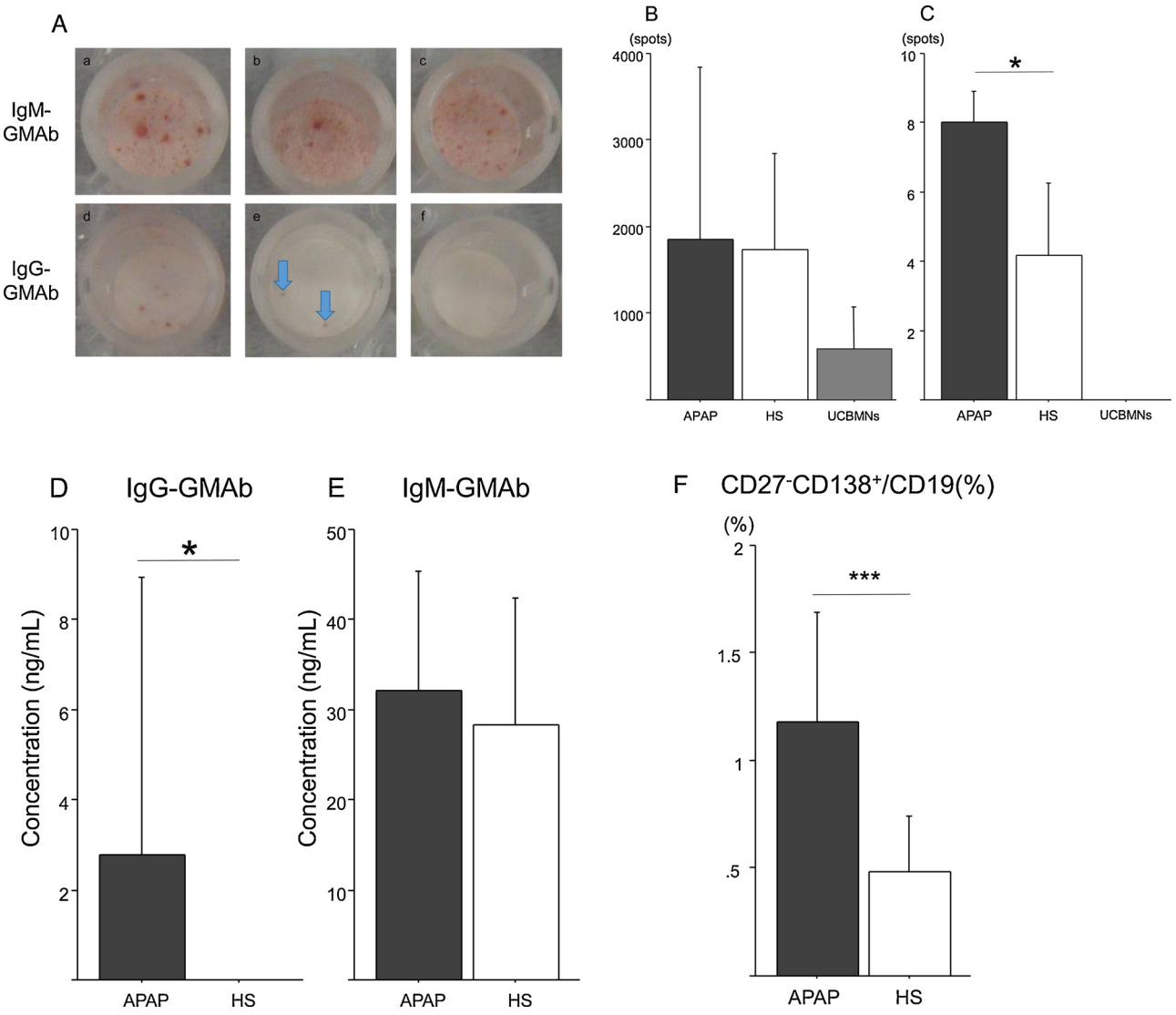

Fig. 2. Detection of PGMPB by EBV-inoculation using ELISPOT assay.
Detection of PGMPB producible cells by EBV-inoculated models using ELISPOT assay. Representative results are shown in Fig. 2**A**. The photos were arranged in order of patient (APAP; **a** and **d**), HS (**b** and **e**), and UCBMNs (**c** and **f**) from the left, and the vertical rows are arranged in order of IgM- and IgG-PGMPB from the top. The blue arrows indicate IgG-PGMPB in a HS.
The spot numbers are shown in Fig. 2B (spot of IgM-PGMPB) and Fig. 2**C (IgG-PGMPB)**. Both panels were arranged in order of patient (APAP, n = 4), HS (n = 3), and UCBMNs (n = 3) from the left. Statistical difference is shown as $*P < 0.05$ compared between patients and HS in right panel. GMAb producible cells from UCBMNs (n = 3) were only IgM-isotype (left panel, gray columns). The vertical axes in the left panel show the spots of producible cells for IgG- or IgM-PGMPB.
The production of IgG- (Fig. 2D) or IgM-GMAb (Fig. 2E) from EBV-inoculated PBMC. The vertical indicates the concentration of GMAb (ng/ml).
The percentages of $CD27^{-}CD138^{+}$ plasma cells in $CD19^{+}$ cells comparing between APAP (n = 19) with HS (n = 21) (Fig. 2F). Statistical difference was observed between APAP and HS (***$p < 0.0001$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

incubated with fluorescent conjugated antibodies, as previously described and according to the manufacturer's instructions, for 30 min on ice in a flow cytometry buffer (pH 7.4 PBS containing 2% FBS and 0.2% sodium azide). After washing twice with the buffer, cells were subjected to flow cytometry analysis using the COULTER EPICS XL Flow Cytometer (Becton Dickinson, San Jose, CA, US and Beckman Coulter, Indianapolis, IN, US). Staining was assessed *via* comparisons with isotype-matched controls. All data were analyzed using FlowJo software (Tree Star, San Carlos, CA, US).

2.9. Statistical tests

Normally distributed data are reported as mean ± SD. Data that are not normally distributed are reported as medians with interquartile ranges [25%, 75%]. Differences among groups were compared using the Mann-Whitney *U* test for all parametric continuous variables. Statistical analyses were performed on a microcomputer using JMP software (SAS Institute, Cary, NC).

3. Results

3.1. In vitro production of GMAb from PBMC stimulated with B cell mitogens

We first evaluated the efficiency of GMAb production from PBMC stimulated with various mitogens *in vitro*. IgG-GMAb was produced exclusively from PBMC that were collected from APAP patients and stimulated with PWM or EBV (Fig. 1A), while IgM-GMAb was produced from PBMC that were collected from both APAP patients and HS and stimulated with CpG or EBV at 2 weeks (Fig. 1B). Since only EBV

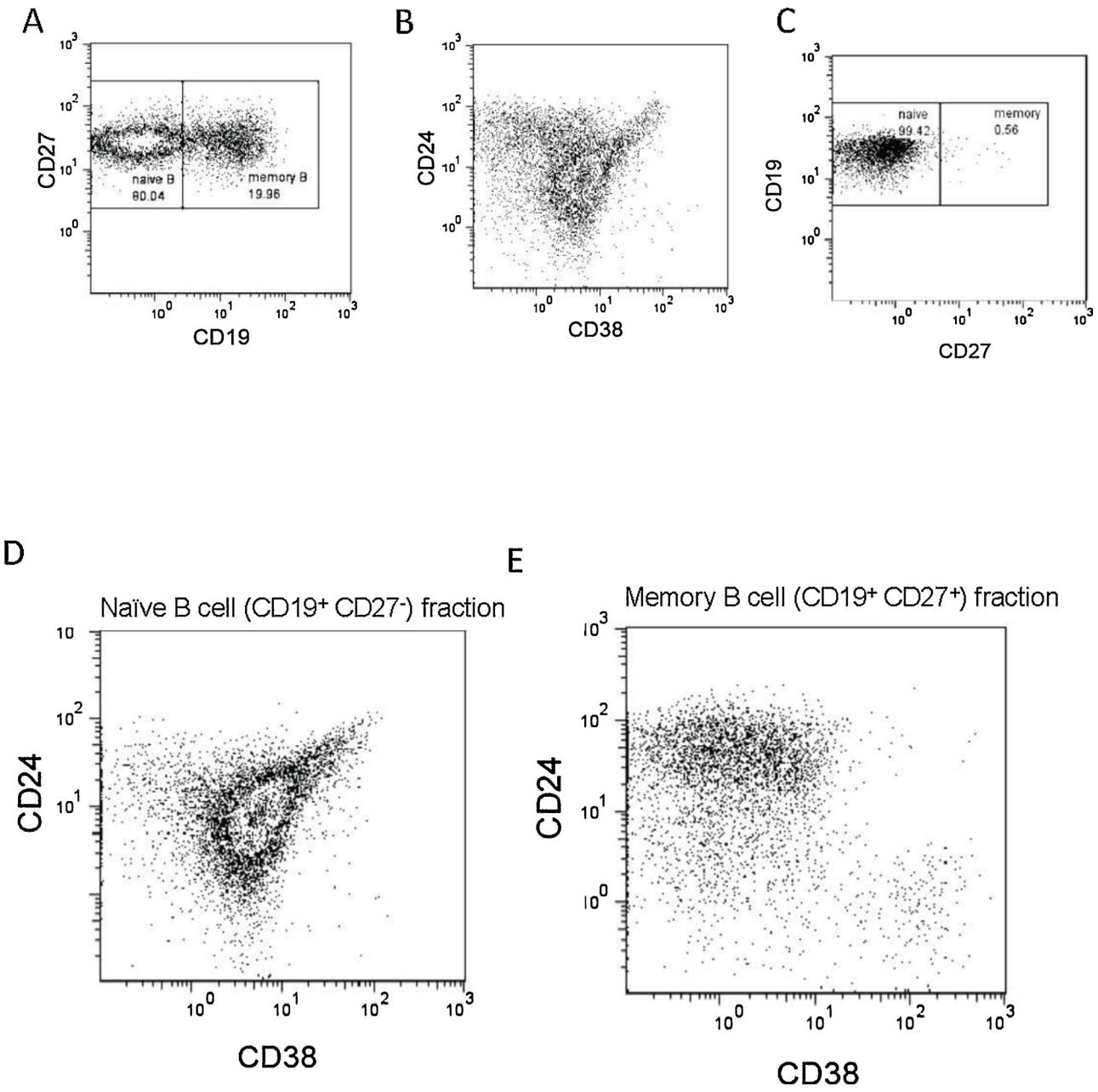

Fig. 3. Analysis of B cell subsets by a flow cytometry. Cells were previously gated on $CD19^+$ cells. Naive and memory B cells were distinguished using CD27 (A) or both CD24 and CD38 (B) expression. The analysis of B cell subsets in UCBMNs for expression of CD19 and CD27 (C). Expression of CD24 and CD38 in anti-CD27 MACS-negative population (D) in the anti-CD27 MACS-positive population (E).

promoted the efficient production of both types of GMAb, we used EBV thereafter as the stimulant for *in vitro* GMAb production. Time-course experiments demonstrated that the concentration of IgM-GMAb in the culture supernatant peaked at 2 weeks for all subjects and to the same extent (32.1 ± 13.3 ng/mL for APAP patients and 28.2 ± 14.1 ng/mL for HS), whereas IgG-GMAb peaked at 3 weeks in the samples from the APAP patients (2.8 ± 6.2 ng/mL; Fig. 1C**, D**), but scarcely increased (0.008 ± 0.009 ng/mL) in the PBMC from HS, and did not increase in the UCBMNs **(**Fig. 1E**)**. These data suggested that IgG-GMAb but not IgM-GMAb production was associated with the disease entity.

3.2. Detection of PGMPB via ELISPOT assay

We next used an ELISPOT assay to quantitate IgM- and IgG-PGMPB directly. As shown in Fig. 2A, 1850 ± 1990, 1735 ± 1110 and 620 ± 390 spots per well for IgM-PGMPB in 1.0×10^5 PBMC were detected in the APAP, HS, and UCBMNs samples, respectively (Fig. 2B). For IgG-PGMPB, 8.0 ± 0.9 spots per well of cells in 1.0×10^5 PBMC were counted in samples from APAP patients and 4.2 ± 2.1 spots per well were observed in those from HS. No IgG-PGMPB spots were found in the UCBMNs ($p < 0.05$; Fig. 2C). The concentrations of IgG- and IgM-GMAb in the culture supernatants were 2.8 ± 6.2 ng/ml and under detection limit for APAP and HS, respectively (Fig. 2D, **E**). Combined with the data from the ELISA, these results show that the enhanced production of IgG-GMAb from PBMC stimulated with EBV occurs as a result of the increased numbers of IgG-PGMPB in APAP patients.

It was observed that there was a discrepancy between the IgG-PGMPB data obtained using ELISA and ELISPOT. In the ELISA, the mean ratio of IgG-GMAb concentration in APAP patient samples to HS samples was more than 10, whereas in the ELISPOT assay, the ratio was 3.92. However, the ratios were similar for IgM-PGMPB: 1.14 and 1.13

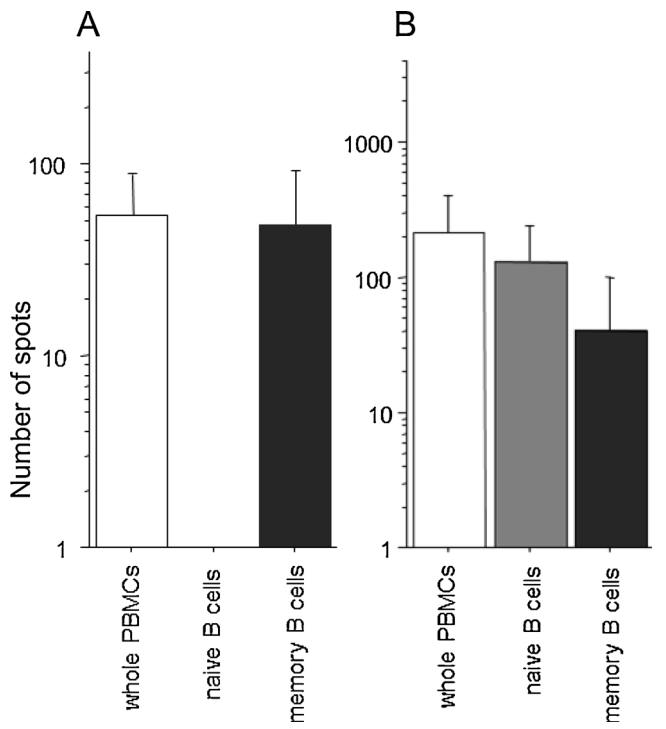

Fig. 4. Detection of IgG-GMAb produsing cells in whole PBMC (white), naive B cell (gray) and memory B cell (black): Fig. 4A, IgM-GMAb: Fig. 4**B**. Data are for a representative APAP case. The vertical axes shows a logarithmic scale of the number of spots per well.

for ELISA and ELISPOT, respectively. This suggests that cellular IgG-GMAbs may be produced more efficiently by IgG-PGMPB from APAP patients than from HS, suggesting that B cell lineage in APAP may be more differentiated than that in HS. To elucidate this idea, we measured the expression of CD27 and CD138 in CD19 positive cells. We found that the percentages of $CD27^-CD138^+$ plasma cells were further higher ($p < 0.0001$, Fig. 2F). Thus, B cell lineage in APAP appeared to move rightward.

3.3. IgG-PGMPB in sorted memory B cells

Because IgG-PGMPB are known to undergo class switching from memory IgM-PGMPB in the germinal center of peripheral lymph nodes, IgG-PGMPB should belong to the memory B cell population. To confirm this, after B cells isolated from APAP patients' blood had been fractionated into memory and naive B cells, IgM- and IgG-PGMPBs were evaluated quantitatively using the ELISPOT assay. There was no rationale for isolating the memory B cell fraction from UCBMNs. Memory B cells ($CD27^+$ B cells/$CD19^+$ B cells) were successfully sorted from the whole Bcell sample, with a purity of > 98% (Fig. 3). The number of spots for IgM-PGMPB in PBMC were 525 ± 367 spots per 10^5 sorted cells. Of those, the memory B cell fraction (71.1 ± 55.3 spots per 10^5 sorted cells) was one-fifth of that in the naive B cell fraction (361.9 ± 292.8 spots per 10^5 sorted cells; Fig. 4), indicating the predominance of naive B cells in the IgM-PGMPB population. On the other hand, all of the spots for IgG-PGMPB (42.9 ± 46.2 spots per 10^5 sorted cells) were in the memory B cell fraction, with similar (37.3 ± 47.6 spots per 10^5 sorted cells) in PBMC and no contribution from the naive B cell fraction (Fig. 4). The mean number of spots for IgG-PGMPB in the memory B cells was significantly higher in samples from APAP patients (131.4 ± 73.8 spots per 10^5 memory B cells) than in those from HS (26.6 ± 26.1 spots per 10^5 memory B cells; Fig. 5, Table 2, $p < 0.05$). This suggests that class switching from IgM-PGMPB to IgG-PGMPB was accelerated in APAP patients relative to HS.

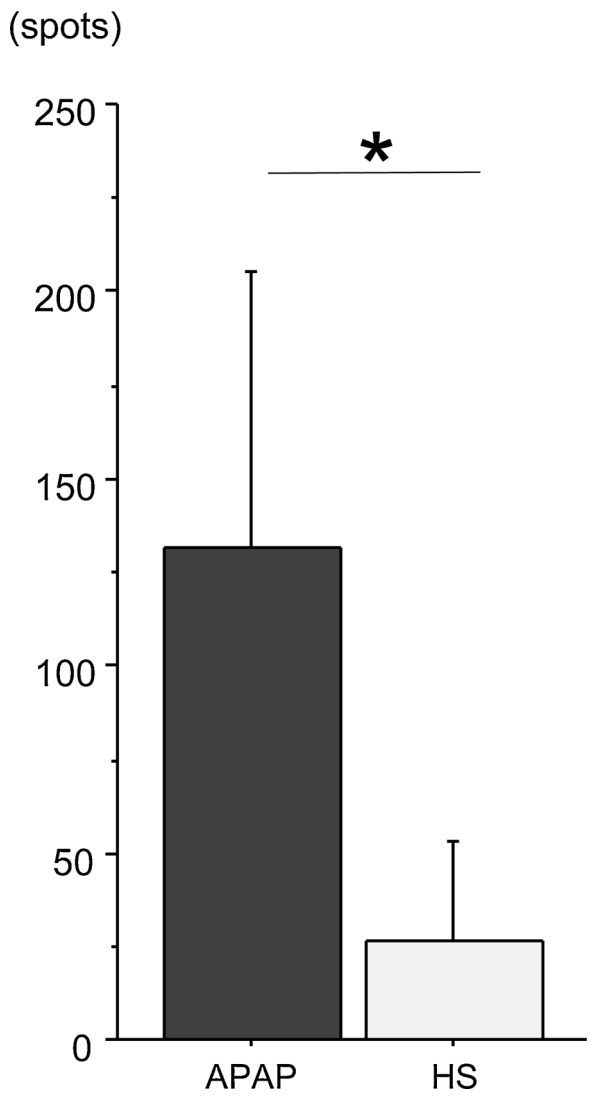

Fig. 5. Comparison of IgG-GMAb producing cells between APAP (n = 5) and HS (n = 5). IgG-PGMPB were detected in 10,000 memory B cells using the ELISPOT assay. Statistical difference is shown as $*P < 0.05$.

4. Discussion

In this study, we found 500–1000 IgM-PGMPB in the PBMC from both APAP patients and HS, but < 30 IgG-PGMPB in the patients' PBMC and hardly any in that from HS. IgG-GMAb production was not detected in EBV-transformed PBMC from UCBMNs, which did not include memory B cells. This supports the idea that IgG-GMAb is produced by memory B cells. This is therefore the first study to quantitatively estimate the number of PGMPB in APAP patients and HS.

The quantity of IgG-GMAb obtained from EBV-transformed APAP patient PBMC was nine-fold higher than that obtained from HS PBMC, according to the ELISA, but only three-fold higher according to the ELISPOT assay. This suggests that the production of IgG-GMAb may be promoted at the cellular level. This discrepancy was intriguing because it did not occur for IgM-PGMPB, suggesting that IgG-GMAb production at the cellular level might be more efficient in APAP patients than in HS. In this regard, our data showed that numbers of $CD138^+$ plasma cells were significantly higher in the APAP patients than in the HS, suggesting that B cell maturation had proceeded in the APAP patients (Fig. 2F) and that EBV-transformed mature IgG-PGMPB might produce higher levels of IgG-GMAb than the EBV-transformed IgG-PGMPB from HS. Recently, Ferretti et al. reported that *Rasgrp1*-deficient mice

Table 2
B cell subset numbers per 100,000 PBMCs and PGMPB.

Category	B cells/100,000 PBMCs		Spot number	
	$CD19^+CD27^+$	$CD19^+CD27^-$	IgG-PGMPB	IgM-PGMPB
aPAP				
patient 1	4472	28628	12	91
patient 2	3240	5430	67	151
patient 3	2123	5947	40	195
patient 4	3280	21660	46	221
patient 5	4874	16226	44	655
healthy control				
control 1	3610	12990	0	78
control 2	2630	11670	14	99
control 3	2180	21820	12	98
control 4	7750	4150	11	152
control 5	3220	9780	3	65

Data were measured using a flowcytemetory. Numbers of spots were enumulated by ELISPOT assay.

produced IgG-GMAb and developed PAP [22]. They also showed that *Rasgrp*1^-/CD275 (ICOSL) $^-$ mice failed to produce IgG-GMAb and thus did not develop PAP, indicating that IgG-GMAb was being produced in a T cell-dependent manner in *Rasgrp*1-deficient mice. In this regard, helper T cells in EBV-transformed PBMC might augment T cell-dependent antibody production *in vitro*.

Recently, we generated a bias-free full-length cDNA library from GM-CSF autoreactive B cells in 10 patients with APAP, amplified Ig-specific PCR amplicons, and performed high-throughput sequencing (HTS; Hashimoto-Takeuchi et al., submitted). In each patient the size of the IgM and IgG clones was similar, and the polyclonality of the IgG isotype was mostly dependent on the variability of naive B cell clones and less so on somatic hypermutation (SHM). Combined with the present results, these findings show that the frequency of IgG-PGMPB is 5% lower than that of IgM-PGMPB, but that the variety of clones is maintained during the differentiation from naive B cells to IgG-PGMPB. However, since the sorted PGMPB did not contain plasma B cells, it was not clear to what degree the polyclonality was maintained at the level of plasma cell maturation, regardless of the polyclonality of serum GMAb.

This study was the first to use the ELISPOT assay to detect B cells producing cytokine autoantibodies. The appropriateness of this method was supported by the fact that spots for IgG-PGMPB, which were expected to be part of the memory B cell fraction, were detected in the sorted memory B cell fraction but not in the naive B cell fraction or in the UCBMNs. In addition, the ELISPOT assay was able to detect extremely low levels of IgG-PGMPB present in PBMC from HS but not from UCBMNs. Since IgG-GMAb is consistently present in the sera of HS as well as in the pharmaceutically prepared IVIG, the small quantity of IgG-PGMPB may eventually differentiate into plasma cells producing IgG-GMAb.

We have provided evidence to support the frequency of IgG-PGMPB was increased in patients with APAP, but this mechanism might not fully explain the excessive production of IgG-GMAb in APAP patients. It is necessary to explain the striking difference in the concentration of serum GMAb between APAP patients and HS, beyond the difference in the density of IgG-PGMPB shown by the ELISPOT assay. As PGMPB differentiate to plasma cells that are major producers of IgG-GMAb, the difference in the frequencies of IgG-PGMPB estimated by ELISPOT assay between the patients and HS may be modest. Considering the classical pathway of antibody production, some IgM-PGMPBs are stimulated by GM-CSF in the germinal center of secondary lymphatic tissues with the help of helper T cells, resulting in class-switching to differentiate into IgG-GMAb producing plasma cells. This hypothesis may be supported by the facts that the frequency of IgG-PGMPBs and the percentages of plasma cells in $CD19^+$ cells were increased in APAP compared with those in HS. In general, antibody production is up-regulated mainly through helper T cells in the germinal center, it is suggested that the presence of helper T cells autoreactive to GM-CSF may be important, although such T cells have not been recognized. Alternatively, attenuation of regulatory T cell function may result in enhanced class-switching and differentiation to plasma cells through dysregulation of the helper T cells. In this regard, chronic suppression of dendritic cell development by GMAb in APAP is likely interfere regulatory T cell maturation [23]. This study focused on the difference of *in vitro* GMAb producing ability from EBV inoculated B cells between APAP and HS. The data suggested increase in IgG-PGMPBs in APAP, but the system used in the study is artificial and may not capture the etiology. Direct observation of GMAb producing plasma cells in animal models or *in vitro* plasma cell culture system may be necessary for future studies.

In conclusion, this study revealed that IgM-PGMPB were consistently present in both APAP patients and in HS, while IgG-PGMPB were exclusively present in APAP patients. Our data supports the idea that IgG-GMAb is produced by memory B cells. We believe that the present study contributes to clarifying the etiology of APAP and may therefore provide avenues for new therapeutic options.

Author contributions

TN, KN, and AH designed this study. TN and KN wrote the manuscript. TN, SU, NM, AH, and CK mainly performed the experiment and analyzed the data. TT and JT assisted in the experiments. RT collected the patient samples and the data. TN, NK, and TT performed the statistical analyses.

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