Hypogammaglobulinemia with a selective delayed recovery in memory B cells and an impaired isotype expression after rituximab administration as an adjuvant to autologous stem cell transplantation for non-Hodgkin lymphoma

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Abstract

Objectives: Some studies have indicated patients who received rituximab as adjuvant to stem cell transplantation had an increased risk of developing severe hypogammaglobulinemia. The mechanism of this hypogammaglobulinemia is unknown, although investigators have hypothesized a further delay in the B-cell recovery as one potential etiology. The aim of this study is to clarify the mechanism(s) of this hypogammaglobulinemia.

Methods: A total of fourteen patients with high-risk $CD20^+$ lymphoma underwent an autologous peripheral blood stem cell transplantation (APBSCT). After a hematological recovery, rituximab was given weekly for up to four doses as an adjuvant therapy.

Results: After a median follow-up of 33.5 months, we found six patients (Group A) who had hypogammaglobulinemia, while the eight other patients (Group B) had normal serum immunoglobulin levels. A phenotypical analysis revealed that Group A patients had already achieved B-cell recovery. However, we found a severe delay in the recovery of $CD27^+$ memory B cells, especially in the $IgD^-/CD27^+$ switched populations in Group A, but CD27 negative naive B-cells reverted to a normal range in both groups. Consistent with this, RT-PCR studies with peripheral blood mononuclear cells revealed that most patients in Group A lacked more than two classes of isotype transcripts.

Conclusions: Abnormal repertoires and impaired isotype expression are seen in patients with common variable immunodeficiency, these data suggested that rituximab after APBSCT can affect not only the B-cell quantities, but also the recovery of the B-cell repertoires.

Introduction

High dose chemotherapy followed by autologous peripheral blood stem cell transplantation (APBSCT) has been widely used for patients with non-Hodgkin's lymphoma (NHL) (1-3). Although APBSCT for patients with high risk aggressive lymphoma may give a prolonged disease free survival (4), relapse is still the primary cause of failure after APBSCT. In addition to residual disease after APBSCT, the reinfusion of tumor cells in the autologous graft may contribute to a relapse. Rituximab, a chimeric anti-CD20 monoclonal antibody, has a good safety profile and, hence, it has been incorporated into many chemotherapeutic regimens and in vivo purging before autologous graft harvest (5, 6). It has been also used as one of the most effective approaches for adjuvant therapy after APBSCT to eradicate the minimal residual disease that leads to relapse (7-9).

Rituximab alone does not appear to cause severe hypogammaglobulinemia according to initial phase I and II clinical trials (10). However, recent studies revealed that patients who received rituximab as an adjuvant to APBSCT had an increased risk of developing severe hypogammaglobulinemia (7, 8, 11). In addition, prolonged hypogammaglobulinemia with rituximab has also been seen in patients with post transplant Epstein-Barr virus associated lymphoproliferative disorder (EBLPD) (12-15) or human immunodeficiency virus (HIV) associated lymphoma (16). The mechanism of this hypogammglobulinemia is unknown, although investigators have hypothesized a delay in the B-cell recovery as one potential etiology.

We previously reported severe persistent panhypogammglobulinemia after rituximab therapy for post transplant Epstein-Barr virus associated autoimmune hemolytic anemia in a Japanese woman (17). We found her B cells to be composed of only CD27 negative naive B cells. In addition, her peripheral blood

mononuclear cells (PBMC) only expressed transcriptions of IgM, IgG1, IgA1 and IgA2 but no IgG2, IgG3 and IgG4. These impaired B-cell repertoires, which are also seen in patients with common variable immunodeficiency (CVID) (18), were speculated to cause her hypogammaglobulinemia. We hypothesized that the same abnormal patterns could be found in the NHL patients treated with APBSCT plus rituximab and therefore performed phenotypical and genetic studies to test our hypothesis.

Patients, materials and methods

Patients and Treatment

From August 2001 to August 2004, a total of 17 Japanese patients with newly diagnosed B-cell NHL were enrolled in our protocol which included APBSCT (5, 19). The eligibility criteria for APBSCT were as follows: age between 15 and 65 years; a confirmed histologic diagnosis of CD20-positive B-cell NHL (excluding Burkitt's lymphoma and lymphoblastic lymphoma); prognostic index, constructed by Coiffier et al (20), of intermediate or high risk as defined by having at least one of the four following adverse features: (1) elevated serum lactic dehydrogenase (LDH) level, (2) bulky mass >10 cm, (3) advanced stage III or IV according to Ann Arbor staging classification, (4) two or more extranodal sites: no severe cardiopulmonary, renal and hepatic dysfunction. After APBSCT, six patients, two who died of NHL and four who were seen by the local physicians, dropped out during the follow-up. Besides these eleven patients, three patients (UPN4, 9 and 13) were enrolled during their first relapse after three to six cycles of CHOP. As a result, a total of fourteen patients were evaluated in this study.

All newly diagnosed patients were initially treated with three cycles of CHOP (21) every two weeks with G-CSF (Lenograstim, Chugai Pharmaceutical CO., Tokyo, Japan) support as induction chemotherapy. One or two leukaphresis were performed after a fourth cycle of CHOP therapy (5). After November 2001, when rituximab was available in Japan, PBSC were mobilized using CHOP plus rituximab (Chugai Pharmaceutical CO., Tokyo, Japan) intended as in vivo purging (5). After the fourth cycle of CHOP, the patients' responses to the initial CHOP were evaluated. While those who achieved tumor reduction rates of more than 50% were treated with two additional cycles of CHOP, those showing less then a 50% response were

considered to be resistant to CHOP and thus were treated with other salvage regimens, including two cycles of DHAP (dexamethasone, cytarabine and cisplatin) (22) or FMD (fludarabine, mitxantrone and dexamethasone) regimens (23). Pre-transplant conditioning regimens for all patients consisted of ranimustine, carboplatin, etoposide and cyclophosphamide (MCVC regimen) (19). After engraftment, rituximab at a dose of 375 mg/m^2 was given weekly for up to four weeks. All patients achieved complete remission after APBSCT. The approval to perform these studies was obtained from the Hokkaido University Hospital's and the Sapporo City General Hospital's institutional review board. Informed consent was provided according to the Declaration of Helsinki protocol.

Cell Preparation

After obtaining the patients' informed consent, blood samples were collected from the patients at the Hokkaido University Hospital with the approval of the ethics committee of Hokkaido University. Peripheral blood mononuclear cells (PBMC) were isolated via density-gradient centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Normal PBMC were obtained from the buffy-coat of blood samples collected from adult volunteers at the Hokkaido Blood Bank.

Flow Cytometry

Three-color flow cytometric immunophenotyping was performed to determine the proportion of lymphocyte subsets. The following monoclonal antibodies specific for human surface antigens were used: anti-CD19-phycoerythrin (PE)-Cy5, anti-CD27-PE, anti-IgD-fluorescein isothiocyanate (FITC), anti-CD3-FITC, anti-CD4-FITC, anti-CD8-PE and anti-CD56-PE were from BD PharMingen (San Diego, CA), and the

appropriate isotype controls from DAKO Japan, (Kyoto, Japan). The cells were stained with saturating amounts of antibodies for 30 minutes at 4^{o} C in Deficient RPMI-1640 (Sigma Aldrich, St. Louis, MO) supplemented with 0.5% bovine serum albumin, washed 2 times, and then were analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA). Flow cytometry data were analyzed using CellQuest^{TM} software (Beckton Dickinson, San Jose, CA). A minimum 50,000 cells per sample were acquired on a flow cytometer.

RT-PCR of immunoglobulin transcripts

Total RNA was isolated from PBMC (5 x 10^{6} cells), using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Subsequently, first-strand cDNA synthesis was performed with SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The primers of the different human immunoglobulin isotypes were used as described previously (18). PCR amplification was performed with reverse-transcribed material and 0.5 U AmpliTaq Gold plymerase (Takara, Japan) with an initial step of 5 min at 95°C; then 40 cycles each consisting of 1 min of extension at 72°C; and at least a final extension for 10 min at 72°C. The reaction products were electrophoresed on 2% agarose gel.

Statistical Analysis

For statistical comparisons between the groups, the Mann-Whiteny U-test was used. Analyses were performed using Glanzman's "Primer of Biostatistics" software package (McGraw-Hill Inc., New York, NY).

Results

Incidence of hypogammaglobulinemia after APBSCT and rituximab

After a median follow-up of 33.5 months (range 12 to 43 months), we found six patients who had hypogammaglobulinemia (Group A), as defined by the serum IgG levels of less than 8.0 g/l (normal range 8.7 to 17.0 g/l), while the other eight patients (Group B) had normal serum IgG levels. There were three follicular lymphoma (FL), two diffuse large cell lymphoma (DLCL) and one mantle cell lymphoma in Group A, and three FL and five DLCL in Group B. The backgrounds of the two groups were not different, including age, the number of CD34 positive cells transplanted, the follow-up period or the dose of rituximab pre- or post-APBSCT (Table 1). We also found the IgA levels to be significantly lower in Group A than Group B (0.46±0.27 g/l and 1.55±0.58 g/l, mean±S.D., $p<0.01$), as well as the IgG levels (5.89±1.72 g/l and 10.9±2.60 g/l) but that was not the case for IgM (0.50±0.39 g/l and 0.85±0.26 g/l).

Absence of memory B cells repertoires in patients who have hypogammaglobulinemia

Since hypogammagloublinemia after APBSCT and rituximab therapy had been hypothesized to arise due to the delayed B-cell recovery, we evaluated the number of B-cells in eleven patients. Unexpectedly, both Group A and Group B patients had already achieved a B-cell recovery to a normal (Group A 135±104 x 10^6/l and Group B 235±113 x 10^6/l, mean±S.D., *N.S.*). Recent studies have shown the CD27 positive memory B-cells to be defective, especially IgD negative switched populations, in some patients with CVID (18). As most patients with CVID have a normal number of B-cells, we hypothesized that the hypogammaglobulinemia seen

after APBSCT and rituximab may therefore be caused by the same mechanism(s) as CVID. To test this hypothesis, we performed a detailed phenotypical analysis on these eleven patients by flow cytometry.

While CD27 negative naive B-cell populations were evident with flow cytometric analysis, $CD27^+$ memory B-cell populations were hardly seen in the PBMC from Group A (Figure 1). The absolute numbers of $IgD^-/CD27^+$ switched memory B cells were as low as 1±0 x 10^6/l, but those of Group B were 15±10 x 10^6/l (mean±S.D., p<0.01, Table 2). The recovery of $CD27^+/IgD^+$ nonswitched memory B cells in Group A was also delayed as well (Group A 2±1 x 10^6/l and Group B 8±6 x 10^6/l, Mean±SD, p<0.05, Table 2), but the number of CD27 negative naive B cells (Group A 130±102 x 10^6/l and Group B 205±103 x 10^6/l, mean±S.D.) recovered to a normal range in both groups (Table 2). In contrast to the B-cell subset, the number and ratio of either $CD4^+$ or $CD8^+$ T cells and $CD56^+$ NK cells did not differ between the two groups (data not shown).

Impaired isotype expression in patients who have hypogammaglobulinemia

The phenotypical analysis suggested that Group A patients had similar abnormal patterns to those with CVID. As a result, we evaluated immunoglobulin isotype production by analyzing the immunoglobulin transcripts by RT-PCR, as patients with CVID are also known to have impaired immunoglobulin class switching (18, 24). Although control donors always expressed each isotype (data not shown), we observed that all patients tested in Group A had defected one or more isotype mRNA transcript expressions in IgG (Table 3). On the other hand, all patients in Group B, except for UPN12 who lacked only IgG4 transcript, showed the normal IgG transcript with RT-PCR. While the IgM mRNA transcript was seen in PBMC of all

patients, the transcript of IgA2 was absent in six out of thirteen tested patients and there appeared to be no differences between the two groups. Taken together with the phenotypical analysis, these data indicate that Group A patients had not only a small number of B-cells, but also abnormal repertories in B-cells.

Bone marrow examinations after APBSCT and rituximab

Bone marrow examinations were performed in nine patients to evaluate any evidence of a relapse. A total 500 cells were evaluated microscopically, and the plasma cells were recognized by their unique morphology. While we found plasma cells (normal range 0.8-1.6%) in all five specimens tested in the Group B patients, only one out of four from Group A specimens showed the existence of plasma cells in the bone marrow. (Table 4)

Clinical complications with hypogammaglobulinemia

UPN1 developed repeated bacterial infections and required the intravenous administrations of immunoglobulin (IVIG). However, the other five patients in Group A, as well as all patients in Group B, have been asymptomatic and required no IVIG.

Discussion

Increasingly, attention is being focused on immune-based therapy for the treatment of NHL. Rituximab is the one of the most frequently administered and well-established monoclonal antibodies which specifically targets CD20 antigen on malignant B cells. CD20 is also expressed on normal B cells, but not on plasma cells, and that has been believed to be the reason why this monoclonal antibody would not cause hypogammaglobulinemia when it was administered as a single agent in earlier studies. However, our survey revealed that six out of fourteen patients (43%) developed hypogammaglboulinemia which was much higher than expected in light of its safety profile when it is used as a single agent.

It has recently been established that CD27 expression could allow for the identification of functional human B cells (25). The impaired B cell subpopulation was noticed in CVID. Piqueras et al proposed a classification based on the quantitative repartition of naive/memory B cells according to the dual expression of IgD and CD27 (18). In their classification, the numbers of $IgD^{+}/CD27^{-}$ naive B cells were not statistically different from those seen in healthy donors, but CD27 positive memory B cells, especially $IgD^{-}/CD27^{+}$ switched memory B cells were significantly decreased. In our cohort, only Group A patients showed the same abnormal patterns of B cells repertories seen in CVID patients (Figure 1 and Table 2).

In addition, the defects of some isotype transcripts in PBMC, which have also been found in patients with CVID, were dominantly seen in Group A (Table 3). But this defect of isotype expression was not specific for Group A, as some patients in Group B also lacked transcripts, especially in IgA2 class. At this moment, it is still unclear as to why the patients in Group A had significantly lower values of IgA than Group B. On the other hand, the transcripts of IgM were evident in all patients.

This suggests that the development of B cells before class switching were maintained in Group A patients. This fact was compatible with the fact that B cells of Group A were composed of mainly naive B cells, which were in the developmental stage before a somatic hypermutation in the germinal center.

Horowitz and colleagues (8) noticed that median IgG levels remained suppressed at less than 10 g/l at 24 months when absolute B-cell number recovered to the normal range in 14 out of 20 patients treated with APBSCT and rituximab. Goldberg et al (26) reported the unusual viral infections in four patients with severe hypogammablobulinemia who were treated with APBSCT and peritransplant rituximab. One patient had a normal number of B-cells when she developed various infections. Since both studies simply showed the whole B-cell counts, whether the same abnormal patterns of B-cells existed in Group A remained unclear. However, it is evident that a small number of B cells could not account for all pathogeneses of hypogammaglobulinemia after APBSCT and rituximab therapy. On the other hand, Lim et al (11) reported hypogammaglobulinemia, especially in IgM, in patients administered ritixumab after APBSCT every 3 months throughout 2-year period. They speculated that a continuous B-cell depletion was the main cause of the severely delayed immunoglobulin recovery. Horowitz and we found a severe reduction of IgG (8) instead of IgM. The schedule of rituxiamb after APBSCT might therefore influence the class of hypogammaglboulinemia.

The reported cases with rituximab related hypogammaglobulinemia were mostly seen in patients who were put under the additional cellular immunosuppression, namely stem cell transplantations (7, 8, 26), organ transplantations (12-14) or HIV infection (16). Moreover, the maintenance rituximab after allogeneic stem cell transplantation was complicated with

hypogammaglobulinemia more often than autologoustransplantation (7). In contrast to Group A patients, we did not see any hypogammaglobulinemia or abnormal B-cell repertoires in five patients who underwent allogeneic transplantation without rituximab in our institution (data not shown). It is believed that allogeneic stem cell transplantation causes patients to demonstrate a more profound immunosuppressive status for prophylaxis of graft versus host disease. However, a simple immunosuppressive therapy without rituximab did not appear to induce the hypogammaglboulinemia with an abnormal repertoire of B cells. This suggests that rituximab played a central role in the imbalanced B-cell recovery. In a normal B cell system, naive B cells that stimulated by surrogate surface B cell receptors with the support of CD40 ligand from T cells differentiate into germinal center B cells, then after somatic hypermutations, they finally become memory B cells or long lived plasma cells. The interaction of these T cells and/or antigen presenting cells and B cells might thus play a role in the pathogenesis of persistence hypogammaglobulinema. Another possible mechanism could be the blocking of maturation capacity in naive B cells which could cause a persistent depletion of the plasma cell populations in bone marrow. This was supported by the persistent depletion of plasma cells in most patients in Group A (Table 4). However, more cases are required to confirm an absence of plasma cells to determine the precise pathogenesis of this hypogammglobulinemia.

In conclusion, our results suggest that rituximab administration after APBSCT cause hypogammaglobulinemia. Although only one patient was symptomatic in our cohort, clinicians should pay extra attention for the possible risk of infections in this immunocompromised situation after high dose chemotherapy. Whether this complication is reversible or not needs to be determined by a longer follow-up. In

addition, whether rituximab administration with less intensive chemotherapy, such as CHOP, can induce hypogammaglobulinemia with abnormal B-cell repertoires should in the future be explored in patients who received ritxumab without APBSCT.

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Figure Legends

Figure 1

Selective loss of $IgD^-/CD27^+$ switched memory B cells after treatment with APBSCT and rituximab.

Peripheral blood mononuclear cells were stained with IgD-FITC, CD27-PE and CD19-PECy5. Naive and memory B cells were defined by membrane expressing IgD and CD27 markers with gating on $CD19^+$ B cells.

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Table 1 Patients' characteristics

	Group A						Group B				
UPN	1	2	3	4	5	6	7	8	9	10	1
Age	65	59	45	50	49	64	63	57	56	44	5
Sex	M	F	F	M	F	M	M	F	F	F	F
Histology	MCL	FL	FL	FL	DLCL	DLCL	FL	DLCL	DLCL	FL	DL
Clinical stage	IIA	IVA	IVA	IVA	IVA	IIIA	IVA	IIIA	IIIA	IVA	IV
Months after APBSCT	42	39	36	17	15	12	43	39	39	34	3
CD34+ cells transplanted ($x10^6$/kg)	2.8	5.1	1.9	3.5	3.2	3.2	2.5	3.0	4.3	4.0	3.
No. of rituximab administration											
Pre-APBSCT	0	1	1	4	6	7	0	0	0	2	2
Post-APBSCT	4	3	3	4	2	1	4	4	4	2	2
Cycles of chemotherapy before APBSCT	6	6	6	9	7	6	7	6	10	6	6
Serum immunoglobulin levels											
IgA (g/l)	0.68	0.25	0.32	0.91	0.37	0.25	1.77	1.40	0.90	1.86	1.1
IgG (g/l)	3.63	7.42	4.77	7.43	4.69	7.42	14,6	10.1	8.01	12.0	10
IgM (g/l)	1.21	0.30	0.21	0.71	0.29	0.30	1.26	0.76	0.89	1.03	0.4

UPN, unique patient number; M, male; F, female; MCL, mantle cell lymphoma; FL, follicular lymphoma
DLCL, diffuese large cell lymphoma; APBSCT, autologous peripheral blood stem cell transplantation

Table 2 B-cell subset reconstitution after APBSCT and rituximab

		$CD19^+$		
UPN	$CD19^+$ ($x10^6$/l)	$CD27^-$($x10^6$/l)	$CD27^+/IgD^+$($x10^6$/l)	$CD27^+/IgD^-$($x10^6$/l)
Group A				
1	131	128	2	1
2	84	81	1	1
3	72	65	3	1
4	334	324	3	2
5	141	138	2	1
6	47	44	2	1
Group B				
7	331	261	20	33
8	189	171	6	12
9	185	173	3	10
10	404	369	12	8
11	332	293	8	14
12	68	60	2	3
13	240	221	5	9
14	134	90	6	28

APBSCT, autologous peripheral blood stem cell transplantation; UPN, unique patient number

Table 3 Isotype expressions in peripheral blood mononuclear cells

UPN	IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
Group A							
1	+	-	+	-	+	+	+
2	+	+	+	-	+	+	-
3	+	+	-	+	+	+	-
4	ND	ND	ND	ND	ND	ND	ND
5	+	+	+	-	+	-	-
6	+	+	+	+	-	+	+
Group B							
7	+	+	+	+	+	+	-
8	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+
11	+	+	+	+	+	+	-
12	+	+	+	+	-	+	-
13	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+

UPN, unique patient number; ND, not done

Table 4 Bone marrow (BM) plasma cells proportions after APBSCT

UPN	% of BM plasm cells	Period of BM examinations after APBSCT
Group A		
1	ND	
2	0.4	14 months
3	0	24 months
4	0	12 months
5	0	6 months
6	ND	
Group B		
7	0.4	7 months
8	ND	
9	3.8	4 months
10	1.2	18 months
11	0.6	26 months
12	0.2	12 months
13	ND	
14	ND	

APBSCT, autologous peripheral blood stem cell transplantation;
UPN, unique patient number; ND, Not done

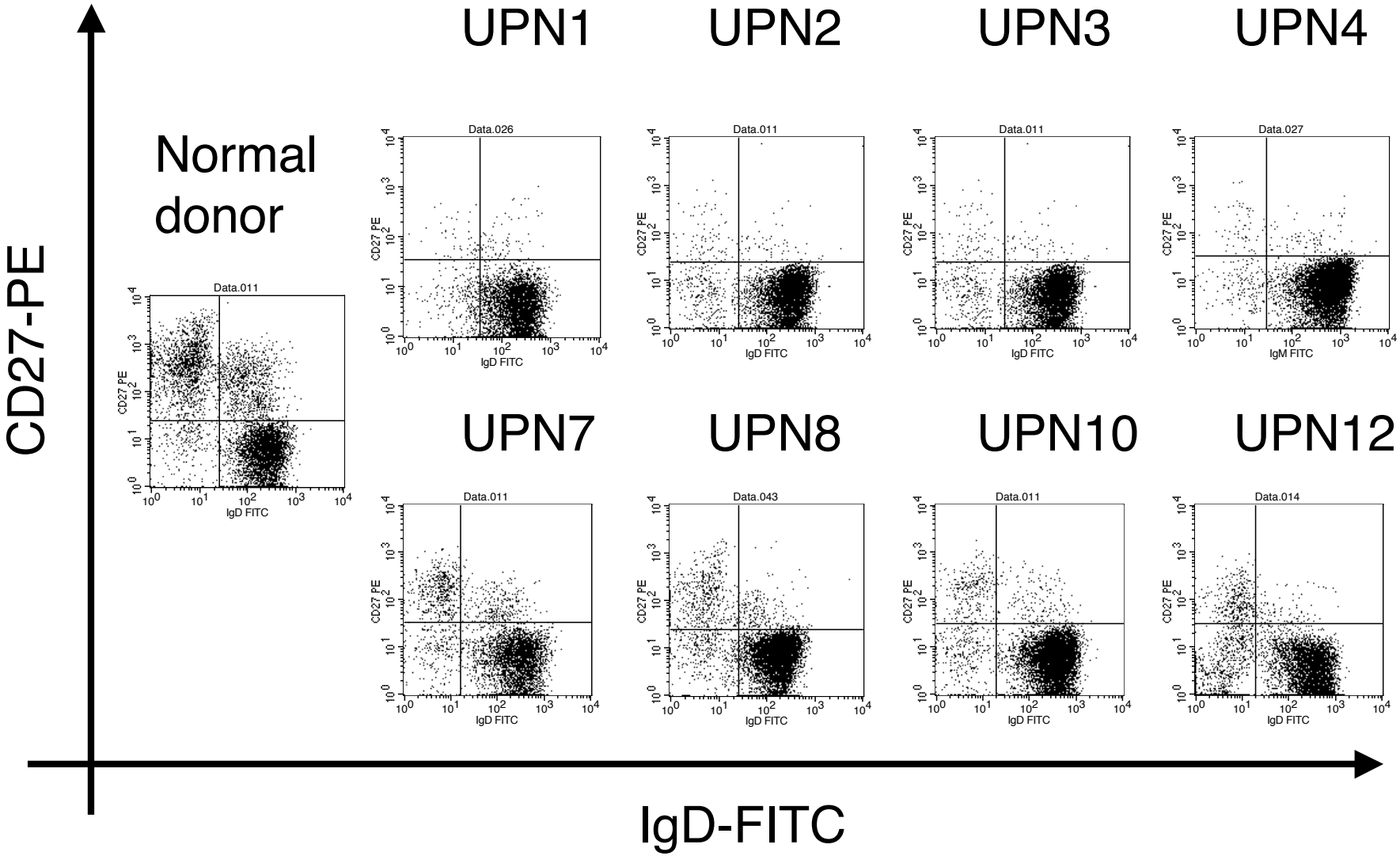
UPN1
UPN2
UPN3
UPN4
Normal donor
UPN7
UPN8
UPN10
UPN12
CD27-PE
IgD-FITC
Data.026
Data.011
Data.027
Data.043
Data.014
CD27 PE
IgD FITC
IgM FITC

Figure 1