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CASE REPORT

Severe hypogammaglobulinemia persisting for 6 years after treatment with rituximab combined chemotherapy due to arrest of B lymphocyte differentiation together with alteration of T lymphocyte homeostasis

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Abstract We report a case of prolonged severe hypogammaglobulinemia after rituximab combined chemotherapy for follicular lymphoma. Although the patient's globulin level was within the normal limits before treatment, the level of IgG dropped below 100 mg/dL, and both IgA and IgM became undetectable after treatment, and the levels have shown no changes for 6 years despite recovery of peripheral B cell counts. Phenotypic analysis of B cells revealed a reduction of class-switched CD27+IgM−IgD− memory B cells below 0.5% and overexpression of CD95. On the other hand, we observed the predominance of memory T cell subsets in both of CD4+ and CD8+ T cells as the result of reduction of naïve T cells. These increased memory T cells overexpressed activation markers such as CD69, CD95, and HLA-DR. Furthermore, the patient's B cells failed to differentiate into memory B or plasma cells in the presence of IL-6, IL-10, IL-15, and BAFF in vitro in comparison with those from healthy controls and showed significant impairment of IgG production. These findings suggest that rituximab combined chemotherapy may induce persistent differentiation arrest and apoptosis of B cell lineage with alteration of T lymphocyte homeostasis resulting in pan-hypogammaglobulinemia.

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1 Introduction

Rituximab is a chimeric monoclonal antibody against the pan-B cell surface marker CD20. Rituximab is widely used in the treatment of B cell malignancies, especially non-Hodgkin lymphoma (NHL) [1, 2]. Although rituximab induces almost complete depletion of normal B lymphocytes in peripheral blood for an average of 6–9 months, hypogammaglobulinemia occurs in only 14% of all cases and it was not considered to be associated with any clinical morbidity. Rituximab alone does not appear to cause severe hypogammaglobulinemia according to initial phase I and II clinical trials. Some recent studies indicated that patients who received rituximab as an adjuvant to autologous stem cell transplantation (ASCT) had an increased risk of developing hypogammaglobulinemia [3–6]. However, in most of these cases, the decreases in globulin levels are mild, and supplementation of intravenous Ig is not necessary. There have been few reports of prolonged hypogammaglobulinemia after rituximab therapy, which is not associated with ASCT [7]. One of these reports concerned HIV-associated lymphoma [8]. In another case exhibiting prolonged hypogammaglobulinemia after chemotherapy with rituximab, the numbers of not only memory B cells but also of CD4+ T cells were decreased. Therefore, fludarabine, which was included in chemotherapy, may affect the immunodeficiency to some extent [9]. Here, we report a case of pan-hypogammaglobulinemia 6 years after completion of treatment combined with rituximab for follicular lymphoma. The patient was HIV-negative, with no family history of immunodeficiency, and the level of

immunoglobulin before treatment was normal. After treatment, the immunoglobulin level was severely and persistently reduced. Phenotypic analysis indicated a predominance of IgD+CD27− naïve B cells and a reduced mature IgD−CD27+ switched memory population, which expressed significantly higher levels of CD95 as compared with healthy controls. In addition, both CD4+ T cells and CD8+ T cells showed diminished CCR7+CD45RA+ naïve T cell subsets, together with predominance of memory T cells, and we observed overexpression of activation marker, such as CD69, CD95, and HLADR, on these T cells. Moreover, CD4+FoxP3+ regulatory T cells were reduced. Based on the data of the present case, rituximab combined chemotherapy was suggested to be a risk factor for persistent differentiation arrest and apoptosis of B cell lineage with alteration of T cell homeostasis, although it may specifically occur in patients with a specific genetic background.

2 Materials and methods

2.1 Clinical samples

In October 2008, blood samples were obtained from the patient and twelve healthy adult donors as control (42 ± 10 years old), after obtaining informed consent.

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll-Hypaque density-gradient centrifugation. B cells were enriched by negative selection from PBMC samples using a MACS direct B cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated B cells was >95%.

2.2 Flow cytometry

PBMCs or enriched B cells were stained with various monoclonal antibody (mAb) combinations for 30 min on ice in staining buffer [1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)]. The directly conjugated antibodies used were anti-IgD-fluorescein isothiocyanate (FITC), anti-CD95-PE, anti-CD69-PE, anti-HLADR-PE, anti-IgG-PE, anti-CD24-PE, anti-CD19-PerCp, anti-CD4-PerCp, anti-CD8-PerCp, anti-CD45RA-allophycocyanin (APC), anti-CD38-APC, anti-CD27-APC (Becton-Dickinson Pharmingen, San Diego, CA), anti-IgM-PE (Serotec, Raleigh, NC), anti-IgA-FITC (Caltag Laboratories, Burlingame, CA), and anti-CCR7 FITC (R&D Systems, Minneapolis, MN). In addition, to assess the CD4+ T cell subsets, we performed intracellular staining of PBMCs. After cell surface staining of CD4-PerCp, cells were fixed and permeabilized with a BD Cytofix/Cytoperm Plus (with GolgiPlug) kit (Becton-Dickinson Immunocytometry) and stained with Fox-P3 Alexia-fluor647 (eBiosciences)

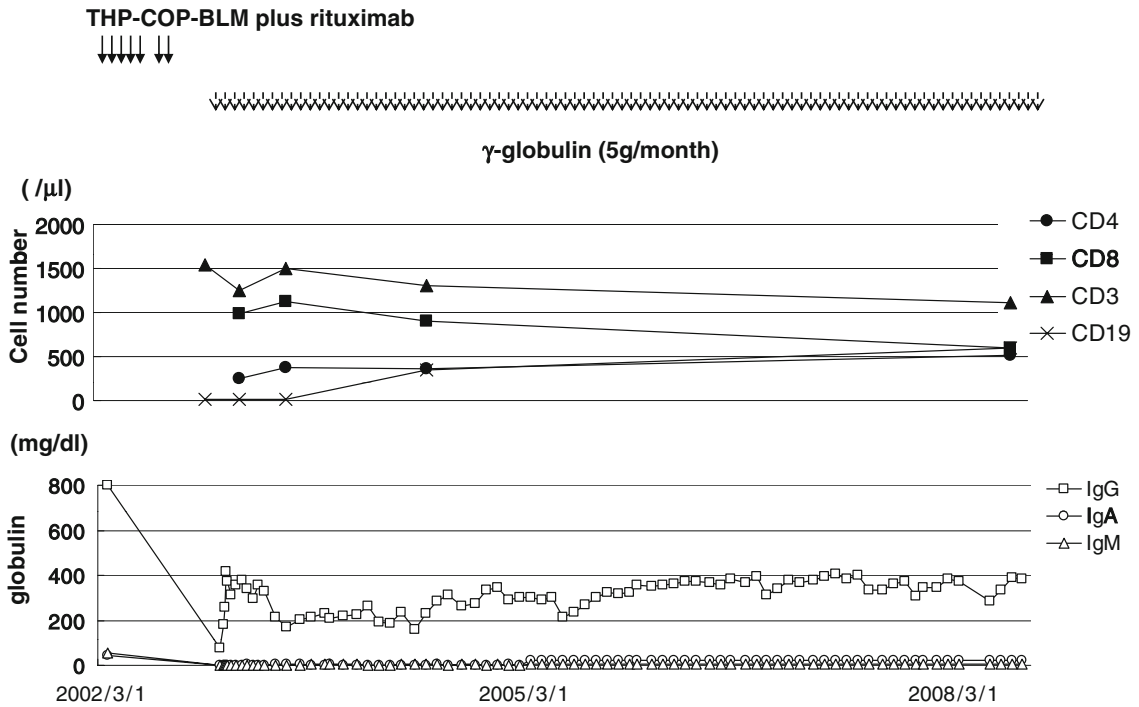

Fig. 1 Levels of immunoglobulins and absolute numbers of lymphocyte subsets over 6 years of follow-up. After rituximab and THP-COP-BLM treatment, the patient showed severe pan-hypogammaglobulinemia. Intravenous immunoglobulin has been administered monthly since then

according to the manufacturer's protocol. Stained cells were washed and data were collected immediately using a 4-color FACScalibur (Becton-Dickinson Immunocytometry) and acquired at least 40000 cells per sample. Data were analyzed using FlowJo software (TreeStar, Stanford University, California).

2.3 B cell differentiation and immunoglobulin assay by enzyme-linked immunosorbent assay (ELISA)

B cell populations were cultured in 96-well plates at 1.5×10^5 cells/mL in 100 μL of culture medium (10% FCS in RPMI medium with L-glutamine, penicillin–streptomycin) alone or supplemented with various cytokines and stimuli: IL-6 (100 ng/mL), IL-10 (25 ng/mL), IL-15 (50 ng/mL), and BAFF (100 ng/mL) or CPG-ODN (0.5 μM) (Invitrogen) for 7 days at 37°C in a humidified atmosphere with 5% CO_2. After 7 days in culture, quantification of IgG and IgM in cell-free supernatants was performed by an ELISA (BRTHYL), and we assessed the B cell subsets by staining with anti-IgD, anti-CD27, and anti-CD38 antibodies.

2.4 Statistical analysis

For statistical comparison between the patient and healthy controls, we assessed the mean ± standard deviation of healthy controls; above and below this range were defined as abnormal levels.

3 Case report

In March 2002, a 60-year-old woman visited Saga Medical School Hospital due to systemic lymphadenopathy. She did not have any family history. A biopsy and subsequent histological examination of the right inguinal lymph node was performed and a diagnosis of follicular lymphoma was made. The clinical stage was defined as stage IV with bone marrow infiltration of lymphoma cells. At diagnosis, serum immunoglobulin levels were as follows: IgG, 800 mg/dL; IgA, 46 mg/dL; IgM, 53 mg/dL. Absolute numbers of CD3, CD4, CD8, and CD19 cells were 394/μL (14.7%), 311/μL (11.6%), 102/μL (3.8%), and 2189/μL (81.7%), respectively. THP-COP-BLM therapy, consisting of pirarubicin (THP-ADR) 40 mg/body, cyclophosphamide (CY) 480 mg/body, vincristine (VCR) 2 mg/body, bleomycin (BLM) 14 mg/body on day 1, prednisolone (PSL) 55 mg on days 1–6, procarbazine (PCZ) 150 mg/body on days 1–10, was started in April 2002. As bone marrow suppression was severe in the first cycle of treatment, the doses were reduced to THP-ADR 30 mg/body, CY 350 mg/body, VCR 1.5 mg/body, PCZ 150 mg/body, respectively, from the second cycle. Rituximab (500 mg/body) was added from the third cycle. Rituximab plus THP-COP-BLM was administered for 5 cycles. Therefore, she underwent a total of eight cycles of chemotherapy until November 2002 and achieved complete remission (CR).

In December 2002, she was referred to Tohoku University Hospital, and found to have severe pan-hypogammaglobulinemia with IgG, IgA, and IgM levels of 74, 0, and 0 mg/dL, respectively. Other laboratory studies revealed WBC 4.2×10^3/μL (Neutro 44%, Lymph 40%, Eo 2%, Mono 14%), lymphocyte subset: CD3 3.8×10^3/μL (91.4%), CD19 <42/μL (<1.0%), RBC 332×10^4/μL, Hb 10.6 g/dL, Ht 33.3%, Plt 22.1×10^3/μL, T-bil 0.5 mg/dL, AST 12 IU/L, ALT 11 IU/L, ALP 417 IU/L, LDH 332 IU/L (normal range in our institute was 119–229), γ-GTP 12 IU/L, ChE 327 IU/L, TP 5.7 g/dL (γ-gl 4.2%), sIL-2R 555 U/mL, CRP 0.8 mg/dL. In terms of T cell subsets, CD4+ cell numbers decreased (254/μL), and the

Table 1 Phenotypic analysis of T and B cells

CD19+ cells			CD4+ T cells			CD8+ T cells		
Subset	Patient	Control	Subset	Patient	Control	Subset	Patient	Control
CD19+/total	6.5	12.5 ± 6.4	CD4+/total	28.5	44.1 ± 7.3	CD8+/total	35	23.4 ± 8.7
IgD+CD27−naïve	95.2	73.7 ± 15.9	CCR7+CD45RA+naïve	14.5	45.4 ± 14.5	CCR7+CD45RA+naïve	5.9	35.2 ± 22.9
IgD−CD27−	0	4.9 ± 2.0	CCR7+CD45RA−TCM	55.8	41.0 ± 11.6	CCR7+CD45RA−TCM	23.9	15.2 ± 9.3
IgD+CD27+	3.4	7.0 ± 10.7	CCR7−CD45RA−TEM	28.3	13.1 ± 3.8	CCR7−CD45RA−TEM	42.7	28.3 ± 16.0
IgD−CD27+	0.5	14.3 ± 6.3	CD69+	8.8	3.0 ± 1.4	CCR7−CD45RA+TEMRA	27.5	21.3 ± 22.2
IgD−CD27high	0	0.3 ± 0.3	CD95+	93.1	59.0 ± 1.3	HLADR+	42.3	6.1 ± 6.1
IgD+CD95+/IgD+	45.7	17.9 ± 9.7	HLADR+	18.9	3.7 ± 2.1			
IgD−CD95+/IgD−	92.9	73.2 ± 8.8	Fox-P3	3.9	5.8 ± 1.6			
Surface IgG	1	7.3 ± 5.7						
Surface IgA	1.5	4.1 ± 1.9						
Surface IgM	82.1	65.7 ± 18.7						

decreased CD4+ cell number resulted in low CD4/CD8 ratio (0.26).

Intravenous immunoglobulin (5 g per month) was begun and has been administered monthly. She experienced herpes zoster in January 2003, but recovered rapidly with acyclovir treatment. As shown in Fig. 1, peripheral B cell count, defined by CD19 positivity, recovered gradually and the percentage rose above the normal limit at the last examination (602/μL, 30.5%). CD4+ T numbers also increased (509/μL) and CD8+ T cell numbers were reduced (596/μL), which resulted in normalization of the CD4/CD8 ratio. The serum concentration of rituximab was examined three times; it was still detectable 7 months after completion of treatment (2.3 μg/mL), but became undetectable 20 months after completion of treatment. As of June 2009, the patient has maintained CR.

3.1 Phenotypic analysis of the patient's B cells

Peripheral B cells were analyzed after rituximab combined chemotherapy for 6 years. Emerging CD19+ B cells showed a predominance of IgD+CD27− naïve B cells and

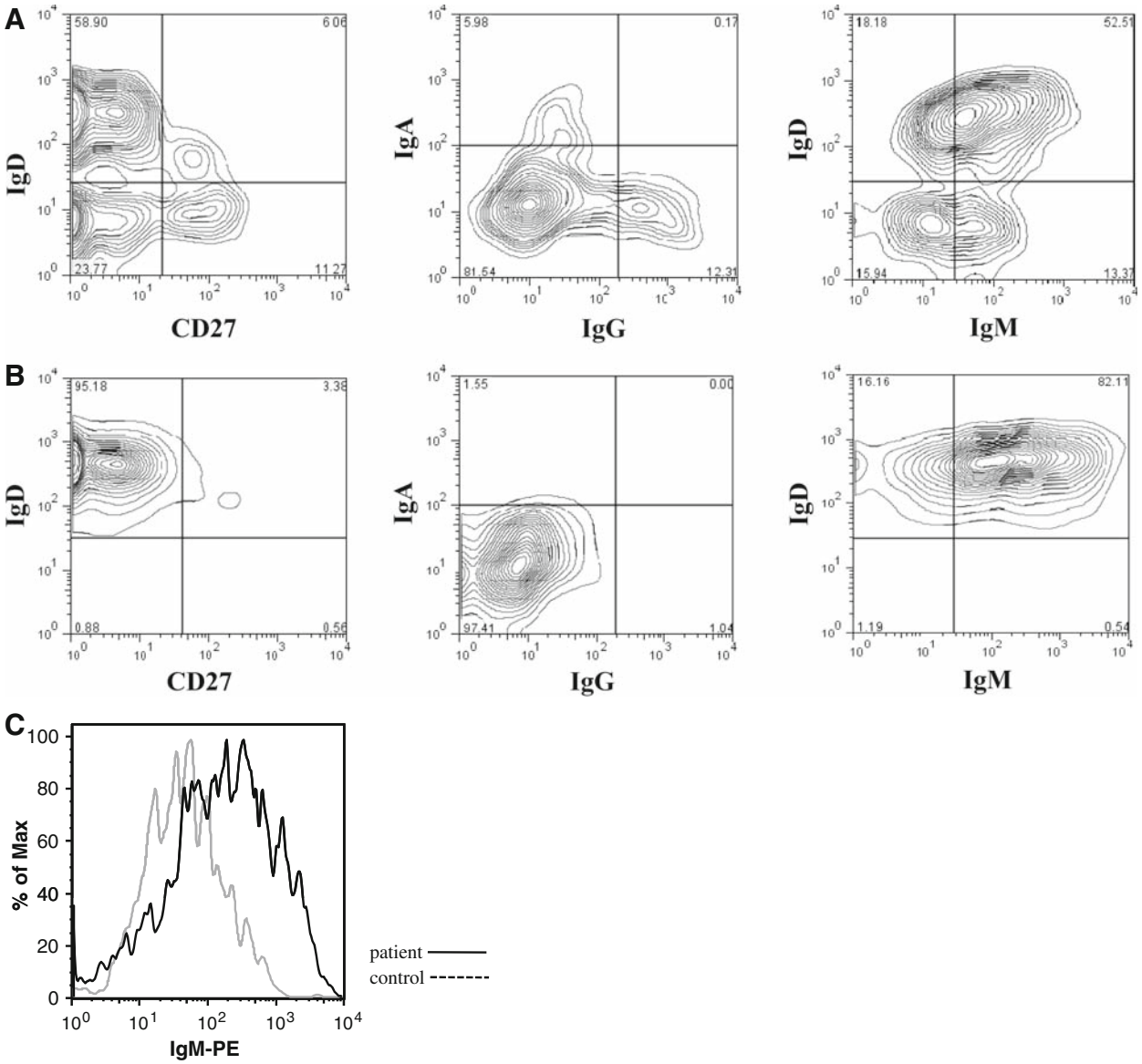

Fig. 2 B cell surface IgG, IgA, and IgM expression. B cells in the patient showed a predominance of IgD+CD27− naïve B cells, markedly reduced IgG and IgA compared with controls (0.71% vs. 7.27 ± 5.74% and 1.34% vs. 4.07 ± 1.91%, respectively): **a** patient and **b** control. **c** The mean fluorescence intensity (MFI) of IgM was above the normal range in the patient's B cells compared with controls (MFI: 113.10 vs. 42.66 ± 3.35). Representative data of the patient and one of the controls are shown

very low levels of memory B cells, with an especially reduced mature IgD−CD27+ switched memory population, although absolute B cell number was not significantly different from the healthy control value (602/μL) (Table 1; Fig. 2). To evaluate whether disappearance of memory B cell after class-switching was specific for our case or not, we analyzed the phenotype of B cells in 5 cases that have normal level of serum immunoglobulin after rituximab contained therapy. In these cases, class-switched CD27+, IgM−, IgD− memory subsets were 9.1 ± 13% which were not significant from healthy controls. Furthermore, the patient's B cells showed markedly reduced surface expression of IgG and IgA compared with controls (1.04% vs. 7.27 ± 5.74% and 1.55% vs. 4.07 ± 1.91%, respectively, Table 1). In addition, we found that B cells showed higher mean fluorescence intensity of cell surface IgM expression compared with controls (MFI: 113.10 vs. 42.66 ± 3.35), although the frequency of the IgM-positive population was within the normal range (81.22% vs. 65.7 ± 18.7%, respectively) (Fig. 2). Moreover, both IgD+ and IgD−B cells from the patient showed significantly elevated CD95 expression compared with controls (45.7% vs. 17.9 ± 9.7% and 92.9% vs. 73.2 ± 8.8%, respectively, Table 1). The frequency of CD38 high CD24 high cells which was characteristic of transitional B cells was within the normal range (data not shown).

3.2 Phenotypic analysis of the patient's T cells

Analysis of CD4+ T and CD8+ T cells in patient revealed diminished CCR7+CD45RA+ naïve T cells of both subsets in comparison with controls (14.5% vs. 45.4 ± 15.4% and 5.9% vs. 35.2 ± 22.9%, respectively, Table 1), together with the predominance of CCR7+CD45RA− central memory CD4+ T cells (TCM) and CCR7−CD45RA− effector memory CD4+ T cells (TEM) compared with controls (55.8% vs. 41.0 ± 11.6% and 28.3% vs. 13.1 ± 3.8%, respectively) (Table 1; Fig. 3). In addition, we observed overexpression of activation markers, such as CD69, CD95, and HLA-DR, on T cells (Table 1). The CD4/CD8 ratio was reduced to below the normal range. The frequency of CD4+FoxP3+ regulatory T cell subsets was reduced in comparison with normal controls (3.9% vs. 5.8 ± 1.6%, respectively, Table 1). We also analyzed the phenotype of T cells in 5 cases that have normal level of serum immunoglobulin after rituximab contained therapy.

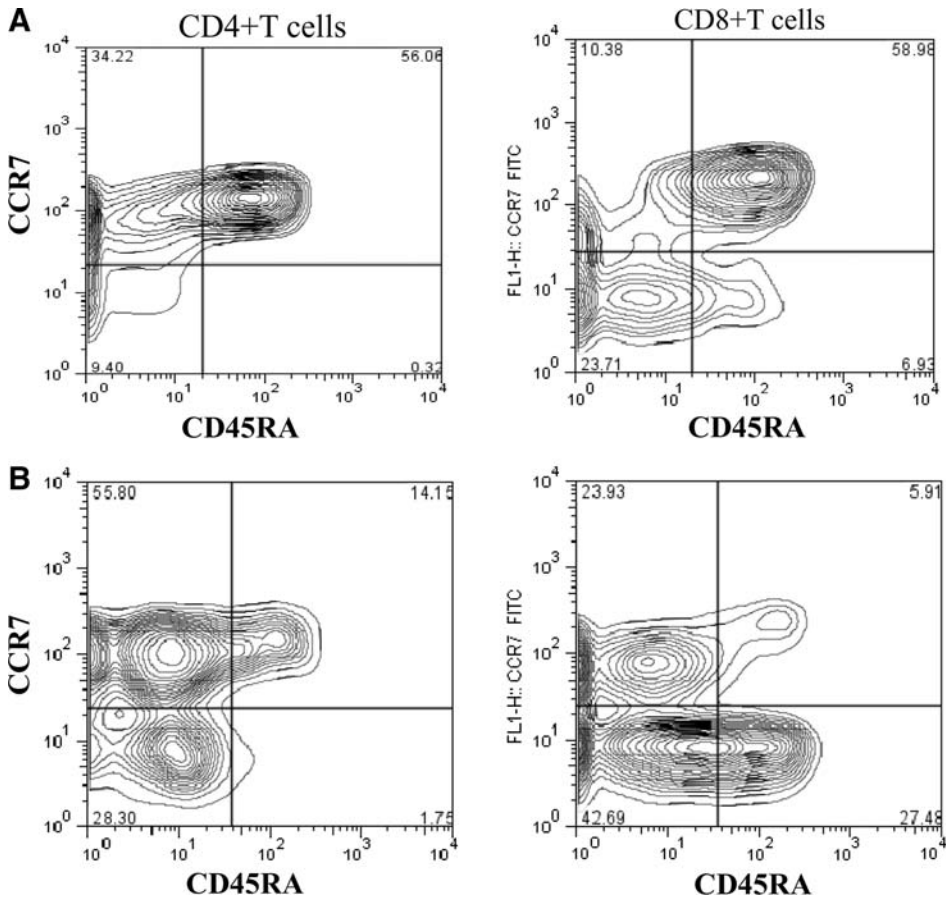

Fig. 3 CD4+ T and CD8+ T cell subsets. Both CD4+ T cells and CD8+ T cells of the patient showed the predominance of CCR7CD45RA memory T cells together with diminished CCR7+CD45RA+ naïve T cell subsets compared with controls: **a** patient and **b** control

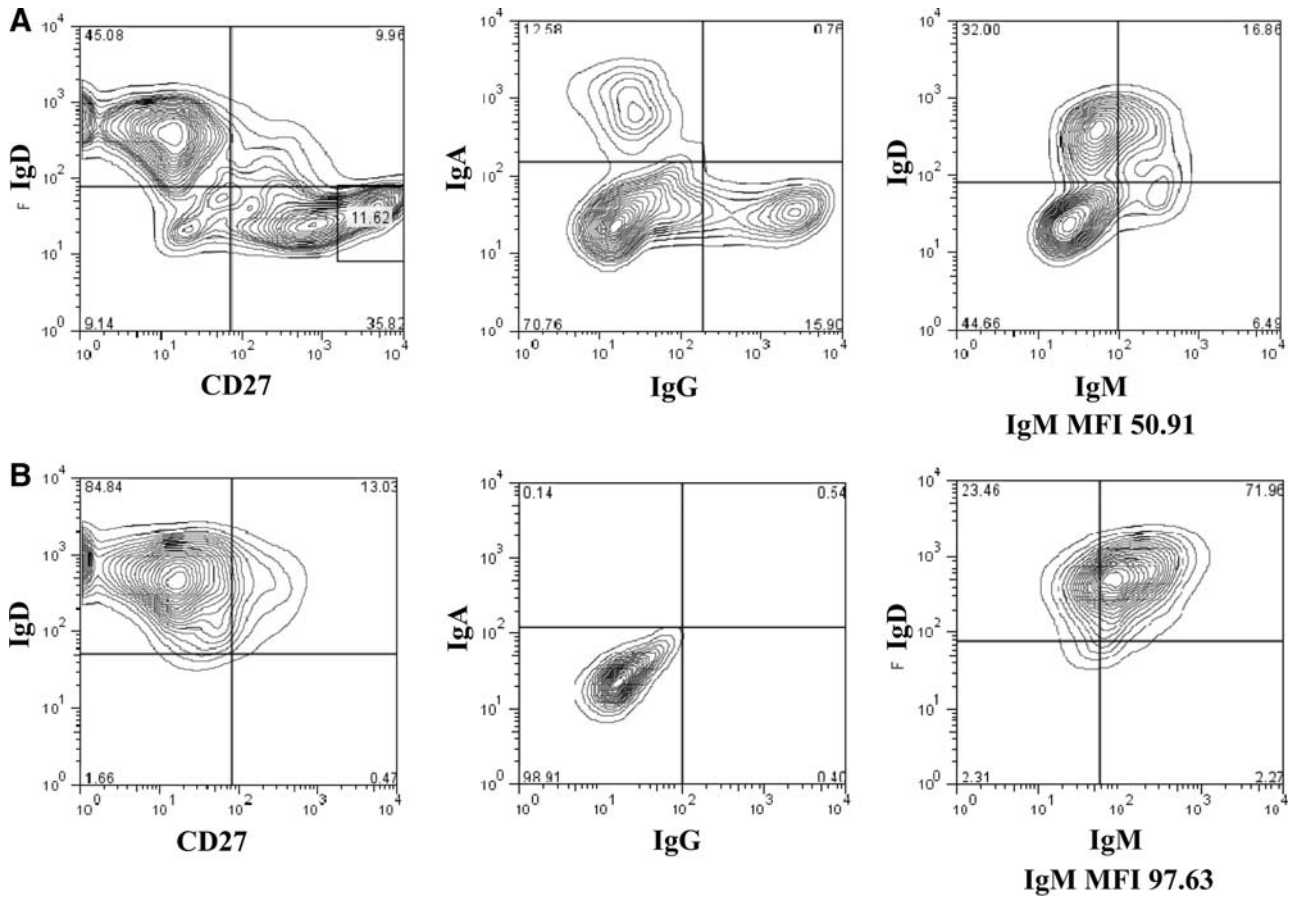

Fig. 4 Phenotypic changes of B cell populations after stimulation with cytokines. B cell populations were cultured in the absence or presence of IL-6, IL-10, IL-15, and BAFF for 7 days. **a** Healthy control B cells: control B cells were partially differentiated into plasma cell population (CD19 low CD27 high, and CD38 high). **b** Patient's B cells: the patient's B cells were almost all naïve B cells at day 0, still maintain IgD+CD27− naïve B subsets (84.8%) together with IgD+CD27+ unswitched memory B cells (13.0%), and did not differentiate into IgD−CD27+ switched memory or plasma cells. Levels of cell surface IgM were elevated on the patient's B cells compared with controls, while neither cell surface IgG nor IgA was expressed

In these cases, T cell subsets were also same as healthy control (data not shown). Similar to the B cell phenotype, we recognized the phenotypic abnormality of the patient's T cells.

3.3 Impaired B cell differentiation and immunoglobulin secretion by in vitro stimulation

B cells from the patient were cultured in the absence or presence of IL-6, IL-10, IL-15, and BAFF for 7 days. Healthy control B cells partially differentiated into a plasma cell population (CD19 low CD27 high, and CD38 high) on stimulation with this cytokine combination. However, the patient's B cells, which were almost all naïve B cells at day 0, still maintained IgD+CD27− naïve B subsets (84.8%) together with IgD+CD27+ unswitched memory B cells (13.0%), and did not differentiate into IgD−CD27+ switched memory or plasma cells. On day 7, cell surface IgM expression was higher on the patient's B cells than controls, while neither IgG nor IgA was expressed on the cell surface (Fig. 4). On day 7, quantification of IgG and IgM in cell-free supernatants was performed by ELISA. Table 2 shows the in vitro production of

Table 2 In vitro differentiation of B cells and immunoglobulin secretion

	Patient (ng/mL)	Normal healthy control, mean ± SD (ng/mL)
IgG		
Nil	54.12	192.00 ± 149.44
IL-6 + IL-10 + IL-15 + BAFF	55.49	903.67 ± 439.72
IgA		
Nil	150.15	188.72 ± 91.38
IL-6 + IL-10 + IL-15 + BAFF	1148.14	1739.29 ± 291.27

IgG and IgA. When stimulated with IL-6, IL-10, IL-15, and BAFF, IgG production was significantly impaired in the patient's B cells compared with controls (55.49 ng/mL vs. 903.67 ± 439.72 ng/mL). On the other hand, although IgA production was also impaired in the patient's B cells in comparison with controls (1148.14 ng/mL vs. 1739.29 ± 291.27 ng/mL), the patient's B cells produced 7.5 times more IgA in the presence of the cytokine combination than IgA in control condition (150.156 ng/mL vs. 188.727 ± 91.3842 ng/mL, respectively).

4 Discussion

As rituximab targets normal B cells as well as neoplastic B cells, almost complete depletion of normal B lymphocytes in peripheral blood was observed for an average of 6–9 months. However, hypogammaglobulinemia related to B cell depletion is not usually problematic, and it was thought not to be associated with any clinical morbidity. Recently, prolonged hypogammaglobulinemia has been reported in some cases receiving rituximab therapy as an adjuvant to ASCT [3, 6, 7]. In most cases, the level of hypogammaglobulinemia is mild, although it sometimes persists for over 2 years. Nishio et al. [10, 11] analyzed the phenotypes of B cells in these cases, and found that recovery of memory B cells was delayed and naïve B cells failed to differentiate into memory cells or plasma cells on stimulation with SAC, IL-2, IL-10, and CD40L in vitro. In addition, they reported that FCγRIIIα gene polymorphism (FCGR3A-158V/F) is related to immunoglobulin level after rituximab therapy as an adjuvant to ASCT. In the present case, we attempted to induce the differentiation of B cells with another cytokine combination consisting of IL-6, IL-10, IL-15, and BAFF. Under these conditions, naïve B cells in our patient also failed to differentiate into memory B cells or plasma cells. Although the reason for B cell differentiation arrest was unclear, there are two possible explanations for these phenomena. First, genetic aberration of a factor essential for immunoglobulin rearrangement may have occurred in lymphoprogenitor cells after combination chemotherapy. Second, rituximab combined chemotherapy may be associated with a risk of persistent differentiation arrest and apoptosis of B cell lineage in patients with a specific genetic background and it may alter the proportion of T cell subsets; this possibility is supported by the increased CD95 (Apo-1, Fas) expression in both B and T cells in lymphoma patients after rituximab treatment. Interestingly, we found abnormality of T cells as well as B cells in this patient. These results suggest that the patient's lymphoprogenitor cells may be destined not to differentiate into Ig-secreting cells by rituximab combined chemotherapy to stem cells.

Previous reports regarding common variable immunodeficiency (CVID) indicated the increased CD95 expression on B and T cells, and a dramatic shift in the patients' T cells to a predominance of memory phenotype rather than naïve type [12–15]. As these results are compatible with the observations in the present case, determination of the alterations of B and T cell subsets by rituximab is important to characterize the mechanism of CVID.

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