

Altered functional B-cell subset populations in patients with chronic fatigue syndrome compared to Healthy Controls

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Abstract

Chronic Fatigue Syndrome (CFS) is a heterogeneous disorder of unknown aetiology characterised by disabling fatigue, headaches, sleep disturbance and several other symptoms. The onset of CFS may follow a viral infection or period of stress. Patients with CFS do not have hypogammaglobulinaemia, predisposition to recurrent bacterial infections or symptoms of autoimmunity. To date, defects in B-cell numbers or function have not been shown in the literature. However, treatment with anti-B-cell therapy using Rituximab has recently shown benefit to CFS patients. We therefore postulated that patients with CFS had a subtle humoral immune dysfunction, and performed extended B-cell immunophenotyping.

We undertook a detailed characterisation of the proportions of the different B-cell subsets in 33 patients with CFS fulfilling the Canadian and Fukuda criteria for CFS and compared these with 24 age and gender matched healthy controls (HC). CFS patients had greater numbers of naïve B-cells as a % lymphocytes - 6.3 % versus 3.9 % in HC ($P=0.034$), greater numbers of naïve B-cells as a % of B-cells - 65 % versus 47 % in controls ($P=0.003$), greater numbers of transitional B-cells - 1.8 % versus 0.8 % in controls ($P=0.025$) and reduced numbers of plasmablasts - 0.5 % versus 0.9 % in controls ($P=0.013$). While the cause of these changes is unclear, we speculate whether they may suggest a subtle tendency to autoimmunity.

Introduction

The present illness called Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) when strictly defined has a prevalence of approximately 0.2 % [1]. The diagnosis of CFS is presently based on patients fulfilling one or more of several criteria [2] [3] [4] [5] and in the absence of an alternative medical or psychiatric cause of the fatigue. The clinical assessment is often supplemented by tests of haematological, biochemical, endocrine and immunological dysfunction as well as investigations checking for inflammation and gluten sensitivity. These tests are nearly always normal or negative.

The aetiology of CFS is still far from understood [6]. Clinicians remain divided as to whether the disease has a physical or psychological cause [7]. Different aetiological hypotheses include viral, immune dysfunction, neurological disease, neuroendocrine disorder, metabolic or autonomic disturbances, ion channel dysfunction, and exposure to toxins or vaccinations, reviewed in [8]. It is of course possible that CFS represents the clinical manifestations of an illness caused by one or more of these factors.

Over the last decade we have observed an elevated prevalence of persistent fatigue in our patients with primary antibody deficiency and speculated on subtle B cell dysfunction in CFS. Recently, 3 patients with CFS were treated with a total of three separate Rituximab infusions which resulted in symptomatic benefit after each infusion [9]. A subsequent larger double-blind, placebo-controlled clinical trial showed symptomatic benefit in 67 % of CFS patients receiving two infusions of Rituximab versus 13 % of CFS patients receiving placebo [10]. These results suggest the involvement of B-cells in the pathology of CFS, in at least in a subset of patients. Importantly, Rituximab does not simply deplete CD20+ cells (B-cells) but has many mechanisms, including down regulating CD40L and CD80 on B-cells, decreasing CD4 effector cells, reducing NK cell numbers and activation, inducing macrophage maturation and reducing TNF α secretion and increasing the suppressive function of T regulatory cells [11]. However, the onset of B-cell depletion did correlate with reduction in symptoms, and with the expected appropriate lag phase. Possible explanations for the improvement of CFS symptoms as a result of B-cell depletion

include allowing repopulation of the B-cell compartment with normalised proportions of subset population, in other words, 'resetting' the B-cell compartment. An alternative explanation includes the removal of auto-reactive B-cells that could be responsible for the pathology of CFS. Additionally, depletion of B-cells may have resulted in the removal of the niche and reservoir of one or more lymphotropic B-cell viruses such as Epstein Barr. It is tempting to favour the removal of auto-reactive B-cells as the female preponderance of CFS is also commonly seen in many autoimmune diseases. In addition, the relapsing/remitting course of CFS is similar to several autoimmune diseases. Moreover, the raised frequency of patient reported lymphadenopathy, sore throat, myalgia and arthralgia that are seen in CFS suggest an inflammatory process [12].

CFS patients do not suffer recurrent bacterial infections like those with primary immune deficiency, but it is possible that subtle defects of B-cell function may underlie CFS. We aimed to characterise the phenotype of B-cell populations in the peripheral blood of CFS patients, and compare that with healthy gender- and age-matched subjects. This approach aimed to determine whether depletion of abnormal B-cells by Rituximab constitutes a mechanism for symptom relief in CFS patients [9, 10]. We also determined serum immunoglobulin concentrations as a basic overall screen of B-cell function. Antibodies are required in an immune response to neutralise and opsonise pathogens and toxic products, and to activate the classical complement system. Defective antibody class switching and antibody production would result in recurrent infection, as seen in primary immunodeficiency states; however, a milder defect may lead to inappropriate immune response and possibly autoimmunity.

Materials and Methods

Subject selection and ethical approval

All participants were verbally informed about the study and given information sheets with written informed consent obtained by ASB. Consecutive patients diagnosed with CFS and fulfilling the Fukada, Canadian and Oxford criteria were selected for study. The Chalder fatigue scale was used to assess tiredness to assign severity. The Hospital Anxiety Depression scale (HADS) and clinical history were used to determine the presence of significant depression and anxiety. Clinically depressed patients (HADS

depression >7) and those with significant anxiety (HADS anxiety >7) were excluded as both are known to affect the immune system. While low mood may be evident in those with CFS owing to the chronic nature of the symptoms, it is distinct from major depression as CFS patients do not generally exhibit the classic symptoms of depression: guilt, anhedonia and low motivation [13-15]. Of 56 eligible CFS patients, 23 were excluded as they were on medication that affected mood, had possible obstructive sleep apnoea, declined participation owing to needle phobia or had CFS with very mild symptoms.

The selection of an appropriate control group in CFS studies is very difficult. Those with depression are often on one or more medications and have increased stress and sleep disturbance that is different to that in CFS. In contrast, patients with impaired mobility often have an underlying illness that affects immune function and are also on a diverse range of medications. We therefore chose HCs who were selected to match the age and gender of our CFS patients. Any volunteer who suffered from chronic illness, depression, anxiety or was on medication was not included in the study. The investigator who performed sample process was unaware if samples were collected from patients or HCs.

This research project was granted ethical approval by the Central London REC 1 Research Ethics Committee (Rec No: 09/H0718/54) and approved by the research and development department at St. Helier hospital, R&D No: 015/2009/DPH.

B-cell immunophenotyping method

5 ml of whole blood was collected into EDTA from each subject and stained immediately following the whole blood method for immunophenotyping B-cell functional subsets as previously described [16]. In brief, 1 ml of whole blood was washed three times in phosphate buffered saline (PBS) with azide and re-suspended in PBS/azide/BSA to a volume of 1 ml. The blood was stained with antibody panels (Table 1) for 20 minutes, lysed with FACS Easy lyse, washed twice in PBS/azide and re-suspended in Cell Fix (Becton Dickinson, Oxford, UK). Samples were acquired on a FACSCalibur cytometer using CELLQuest software (Becton Dickinson, Oxford, UK) (Figure 1 and 2).

Measurement of immunoglobulin concentrations

Clotted samples were also taken and serum was separated and stored at – 80 °C. Sera were thawed and immunoglobulin concentrations were measured for all of the samples using a rate nephelometer (BN2 from Dade Behring) on the same day.

Data and statistical analysis

All of the data was determined to be non-parametrically distributed during analysis using the Kolmogorov-Smirnov normality test except the B-cells as a percentage of lymphocytes. The data was therefore analysed using the non-parametric Mann-Whitney Rank Sum test. The data was statistically analysed on two separate occasions using two software packages: SigmaStat version 3.11 and by SPSS version 20. Identical statistical results were obtained using both packages. The P values were not modified.

Results

The demographics of participants are summarised in table 2. The majority of the CFS patients were of moderate severity (23 patients) as assessed by the Chalder Fatigue Scale. Two patients were severely affected and the rest were mild (8 patients). For most B-cell populations measured, the ranges of both patients and controls were quite wide. This was expected, as B-cells are a dynamic immune population. B-cell populations fluctuate in response to many parameters, such as infection and stress, and so tight clusters of data were not expected. The majority of B-cell subset populations showed no significant difference in patients versus controls, subsets that were significantly different are highlighted in light grey (Table 3), and shown in (Figure 3). CFS patients had greater numbers of naïve B-cells as a % lymphocytes - 6.3 % versus 3.9 % in HC (P=0.034), greater numbers of naïve B-cells as a % of B-cells - 65 % versus 47 % in controls (P=0.003), greater numbers of transitional B-cells - 1.8 % versus 0.8 % in controls (P=0.025) and reduced numbers of plasmablasts - 0.5 % versus 0.9 % in controls (P=0.013).

Immunoglobulin concentrations

The immunoglobulin concentrations of the CFS patients and controls were normal, as measured by rate nephelometry (Table 4). This was expected as neither of the groups reported recurrent infections.

Application of the CVID classification to CFS patients

Owing to their tendency to 'recurrent infections', some patient groups have suggested that those with CFS have some sort of immune deficiency syndrome. With this in mind the B-cell characterisation data were used to determine classifications of CFS patients and controls along the lines of those with CVID using the Warnatz [17], Paris [18] and EUROclass [19] definitions (Table 5). These definitions are used to assign CVID patients into groups that are thought more likely to develop complications such as lymphadenopathy, splenomegaly, granulomas and autoimmunity. CFS patients do not classically suffer from splenomegaly, granulomas or significant autoimmunity and so it was expected that this analysis would show no difference between the CFS patients and controls. Indeed, there was no significant difference between the CFS patients and controls regarding classification, as determined by Chi Squared test analysis.

Discussion

We selected a well-defined group of CFS patients and a group of age, sex and ethnicity matched controls. We detected no difference in the immunoglobulin concentrations. However, compared to the HC the CFS cohort had significantly greater proportions of transitional B-cells, naïve B-cells and reduced proportions of plasmablasts expressed as a percentage of B-cells.

CFS and B-cells

B-cells produce antibodies and are potent antigen presenting cells. Impairment of B-cell function or development leads to recurrent infections, or a propensity to autoimmunity or allergy. The majority of early studies on CFS patients found no difference in B-cell numbers or immunoglobulin concentrations compared with HCs [20, 21]. Interestingly, One study did find increased B cell lymphocyte numbers in CFS patients, with a subset of these patients (37%) having an expansion of CD5+CD19+ B cells. These latter cells have been considered producers of IgA, with

increased numbers putatively involved in autoimmune disease [22]. However, all of these studies were published before the new CFS case definitions were proposed, thus our CFS cohort is not directly comparable to these studies. Regardless, CFS patients do not suffer from significant recurrent upper or lower respiratory tract bacterial infections, so it is unlikely that CFS patients have profound B-cell abnormalities. However, immune abnormalities have been suggested by the results of a large epidemiological study, which looked at 1.2 million cancer cases and 100,000 controls [23]. This study found that CFS was associated with an increased risk of Non-Hodgkins lymphoma (NHL) in elderly patients [23]. The authors speculated that the increased risk of NHL attributable to CFS may indicate chronic immune activation or infection. Specifically, the NHL subtype most associated with CFS was large B cell lymphoma which may indicate problems with B cell development but more research is required in this area. Moreover, patients with CFS have been shown to benefit from treatment with repeated infusions with Rituximab [9, 10]. While the exact mechanism of the delayed symptomatic benefit from the infusions is unclear, it is possible that it is related to the elimination of dysfunctional B-cell populations [10]. Thus CFS patients may have some unusual, unrecognised autoimmune disease, or it is possible that CFS patients are unable to control lymphotropic viral infections due to some defect of B-cell memory or T-cell dysfunction. As far as we are aware extended B-cell immunophenotyping has not been reported on the CFS patients that received Rituximab. Our earlier observation of persistent fatigue in those with primary antibody deficiency supported by the beneficial effect of Rituximab in CFS prompted us to specifically search for a subtle defect in B-cell subsets, which would not have previously been detected by a simple analysis of B-cell numbers.

B-cell development and transitional B-cells

B-cell development in the bone marrow is antigen independent and is a tightly regulated process [24]. After several rounds of expansion, functional light chains replace the surrogate light chain and pair with the μ heavy chain resulting in cell surface IgM expression and forming the B-cell receptor (BCR). BCR expression allows negative selection of autoreactive B and their elimination by apoptosis. Deficiency of negative selection that eliminates cells with autoreactive heavy chains may allow the later development of systemic autoimmunity [25]. Surviving B-cells subsequently become transitional B-cells (CD19+CD38++IgM+IgD+) and migrate

via the peripheral blood into secondary lymphoid organs such as the spleen, where full maturation occurs [26]. Only 10-20 % of immature B-cells produced in the bone marrow reach the spleen, a large proportion are deleted due to the expression of autoreactive BCRs. Our CFS patients had increased transitional B-cells, so it is tempting to speculate whether this reflects a defective negative selection checkpoint. If so the consequence of this defect may encourage self-reactive B-cells to escape the bone marrow. Importantly, increased numbers of transitional B-cells have been reported in several patient groups with defective humoral immunity, including patients with SLE [27] [28], X-linked Lymphoproliferative disease, CVID, patients recovering from haemopoietic transplantation and neonates [29].

B-cell development in the spleen and naïve B-cells

In the spleen transitional B-cells mature into long lived naïve B-cells (CD19+IgM+IgD+CD27-) and an important survival factor is BAFF (Blys) and its receptor BAFF-R. The CFS patients had greater proportions of naïve B-cells compared to controls which is not surprising as they have greater proportions of transitional B-cells which are naïve B-cell progenitors. Nonetheless, it is also possible that CFS patients may be producing more BAFF to allow the survival of these increased proportions of naïve B-cells. Once through this transitional stage of development, B-cells develop into either follicular B-cells (CD19+CD27-CD38+mIgD+mIgM^{hi}) or marginal zone (MZ) B-cells (CD19+CD27+CD38-mIgD+mIgM^{hi}).

Plasmablasts

Antigen encounter stimulates affinity maturation of the BCR by somatic hypermutation within germinal centres. During this process, follicular B-cells can differentiate into memory B-cells (CD19+CD27+CD38+mIgM-mIgD-) or plasmablasts (CD19+CD27++CD38^{hi}) while MZ B-cells only differentiate into plasmablasts. Memory B-cells can be subdivided into non-switched memory B-cells (CD19+CD27+IgM+IgD+) or class-switched memory B-cells (CD19+CD27+IgM+IgD-) depending in part, on the cytokine milieu. In CFS patients, memory B-cell subsets were comparable to controls, but plasmablasts were reduced. One explanation for reduced plasmablasts in the CFS cohort is that increased numbers of transitional B-cells and naïve B-cells may overwhelm the B-cell maturation

process, which may consequently become suboptimal. Alternatively T-cell help provided by cytokines may not support naïve B-cells to develop into plasmablasts.

CFS patients and altered B-cell subsets

In patients with CFS it is possible that there are one or more alterations of B-cell maturation which may lead to an increased tendency to autoimmunity and a subtle humoral immune dysfunction. There are no studies detailing B cell populations in patients with depression and significant anxiety. B-cell negative selection requires appropriate integration of complex signals that are also key for clonal expansion, isotype switching, affinity maturation, long lived memory, and maintaining tolerance to self-antigens. At least two broad categories of genetic defects promote loss of B-cell tolerance and promote autoreactivity. If B-cell thresholds for cellular signalling, activation, or proliferation are altered this will increase the risk of self-reactive B-cells escaping the checkpoints and the potential of autoimmune disease. Additionally, defective apoptotic genes increase B-cell life spans, allowing survival of self-reactive B-cell clones, leading to the potential of autoantibody production and possible autoimmune disease. There are a small number of studies showing an increased frequency of low level auto-antibodies directed against nuclear, thyroid and other antigens in CFS patients [30, 31]. Others have proposed autoimmunity to neuropeptide antigens [32]. Alternatively, depletion of B-cells may have resulted in the removal of the reservoir of lymphotropic B-cell viruses such as EBV. However, the clinical improvement of patients with CFS after Rituximab treatment suggests an active process by which the dysfunctional B-cell maturation process contributes to symptomatology. It would be interesting to monitor B-cell subsets of CFS patients before and after Rituximab treatment and monitor repopulation of the different B-cell subsets with the return of symptoms.

CFS patients do not experience splenomegaly or granulomatous disease, but some complain of lymphadenopathy. Thus the B-cell characterisation of the CFS patients was used to assess whether CFS patients exhibit similar B-cell subset abnormality patterns to the CVID patients, and whether there is a significant difference with controls (Table 3.5). Intriguingly, the CFS patients showed increased transitional B-cells compared to controls (1.9 % of B-cells in CFS group compared to 0.8 % of B-cells in HC group, $P=0.025$). In the CVID patient cohort, increased transitional B-

cells ($\geq 9\%$ of CD19+ B-cells) are associated with lymphadenopathy, a pathology that CFS patients commonly experience. A reported normal range of transitional B-cells as a percentage of B-cells is 0.6-3.4 % of B-cells [33] and so the CFS cohort in this study had only a modest expansion of transitional B-cells compared to controls. However, the CFS patients examined in our cohort were mainly moderate sufferers with intermittent but not persistent lymphadenopathy. It is tempting to speculate whether a group of severe CFS patients suffering from more frequent or significant lymphadenopathy may have an even greater increase of their transitional B-cells perhaps, similar to that in the CVID cohort.

In conclusion we have observed patients with moderate CFS to have increased proportions of transitional and naïve B-cells and reduced plasmablasts. The precise basis for these findings is unclear and our work does not allow clarification of whether these changes are the cause of the CFS symptoms or the result of patient inactivity, sleep disturbance or raised stress. The therapeutic response to Rituximab suggests that B-cells are somehow involved in the pathogenesis or perpetuation of CFS symptoms.

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Contribution

AB designed experiments. ASB carried out laboratory work. BF assisted with technical difficulties. ASB analysed results. AB, ASB and BF discussed results. ASB wrote the manuscript and AB edited the manuscript. BF reviewed the manuscript.

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Conflict of interests

The authors have no conflict of interests to declare.

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Table 1 – The antibody panel used to stain the whole blood.

Tube	Antibody combinations			
1	CD19-Pcy5	CD27-FITC	IgD-PE	IgM-Cy5
2	CD19-Pcy5	CD21-FITC	CD38-PE	IgM-Cy5
3	CD19-Pcy5	IgG1/2A-FITC		

Table 2 –Demographics of patients and HC enrolled in the study.

Parameter	CFS patients (<i>n</i> =33)			HC (<i>n</i> =24)			P value
	Mean	Range	SD	Mean	Range	SD	
Age (year)	35	20 – 66	11.8	40	22 – 63	12.2	0.183
% Female	79 %			86 %			N/A

Table 3 – Functional B-cell subsets in CFS patients versus HC

Cell population compared	CFS patients (n=33)				HCs (n=24)				% diff	P value
	Min-Max	Med.	25 % quartile	75 % quartile	Min-Max	Med.	25 % quartile	75 % quartile		
B-cells (% lymphocytes)	3.4-23.2	9.5	6.5	12.8	1.8-19	10.3	6.4	13.9	-8	0.802
Switched memory B-cells (% lymphocytes)	0.0-5.6	0.4	0.0	0.9	0.0-2.9	0.5	0.2	1.0	-20	0.167
Switched memory B-cells (% B-cells)	0.0-28.0	5.2	0.0	7.7	0.0-29.0	6.9	3.6	11.9	-25	0.082
Marginal Zone B-cells (% lymphocytes)	0.2-2.6	0.99	0.7	1.6	0.1-5.7	1.2	0.8	1.4	-18	0.853
Marginal Zone B-cells (% B-cells)	3.3-25.8	11.0	8.1	16.2	3.0-34.3	11.4	7.5	17.3	-4	0.740
Naïve-B-cells (% lymphocytes)	1.7-16.3	6.3	4.2	7.9	0.1-12.9	3.9	2.0	6.7	138	0.034
Naïve-B-cells (% B-cells)	33.8-85.0	65.0	52.8	71.5	0.3-77.6	47.0	32.8	62.9	128	0.003
CD38low CD21low cells (% B-cells)	0.6-20.3	2.7	1.1	4.4	0.1-68.3	3.1	2.4	4.1	-13	0.193
Transitional B-cells (% B-cells)	0.0-8.3	1.8	0.5	4.1	0.0-7.3	0.8	0.3	1.2	155	0.025
Plasmablasts (% B-cells)	0.0-8.6	0.5	0.3	1.1	0.1-24.3	0.9	0.6	2.7	-44	0.013
CD27+ cells (% B-cells)	12.6-45.8	25.2	19.5	37.5	13.6-97.0	27.4	19.3	46.0	-8	0.268
non-switched memory B-cells (% B-cells)	5.0-27.6	13.3	9.5	18.0	7.1-97.0	14.5	11.6	24.8	-8	0.162

Results of the statistical analysis of B-cell functional subsets using the non-parametric Mann-Whitney Rank Sum test. P < 0.05 represent a statistically significant result, these results are highlighted in light grey. The statistical data has not been modified. Med – median.

Table 4 – Immunoglobulins (Ig) concentrations of CFS patients and controls. IgG and IgM were parametrically distributed, so were analysed using a T test. IgA was non-parametrically distributed and was analysed using a Mann Whitney U test.

Ig	CFS patients (n=33) (g/L)			HCs (n=24) (g/L)			P value
	Min-Max	Mean	SD	Min-Max	Mean	SD	
IgG	7.3-16.1	11.1	2.22	7.6-15.3	11.1	1.85	0.94
IgM	0.5-3.7	1.4	0.6	0.5-2.3	1.3	0.5	0.57
	Min-Max	Med	25% & 75% quartile	Min-Max	Med	25% & 75% quartile	P value
IgA	0.8-5.5	1.8	1.4 & 2.3	0.8-4.1	2.2	1.5 & 2.5	0.251

Table 5 – The Classification of the CFS patients and controls using the Warnatz, Paris and EUROclass definitions.

	WARNATZ	PARIS	EUROCLASS
CFS patients (n=33)	II = 15 (45%) Ib = 18 (55%)	MB1 = 25 (76%) MB2 = 8 (24%)	SmB(+)21(norm) = 19 (58%) SmB(-)Tr(norm)21(norm) = 13 (39%) SmB(+)21(low) = 1 (3%)
HCs (n=24)	II = 15 (63%) Ib = 9 (38%)	MB1 = 14 (58%) MB2 = 10 (42%)	SmB(+)21(norm) = 18 (79%) SmB(-)Tr(norm)21(norm) = 4 (13%) SmB(+)21(low) = 2 (8%)

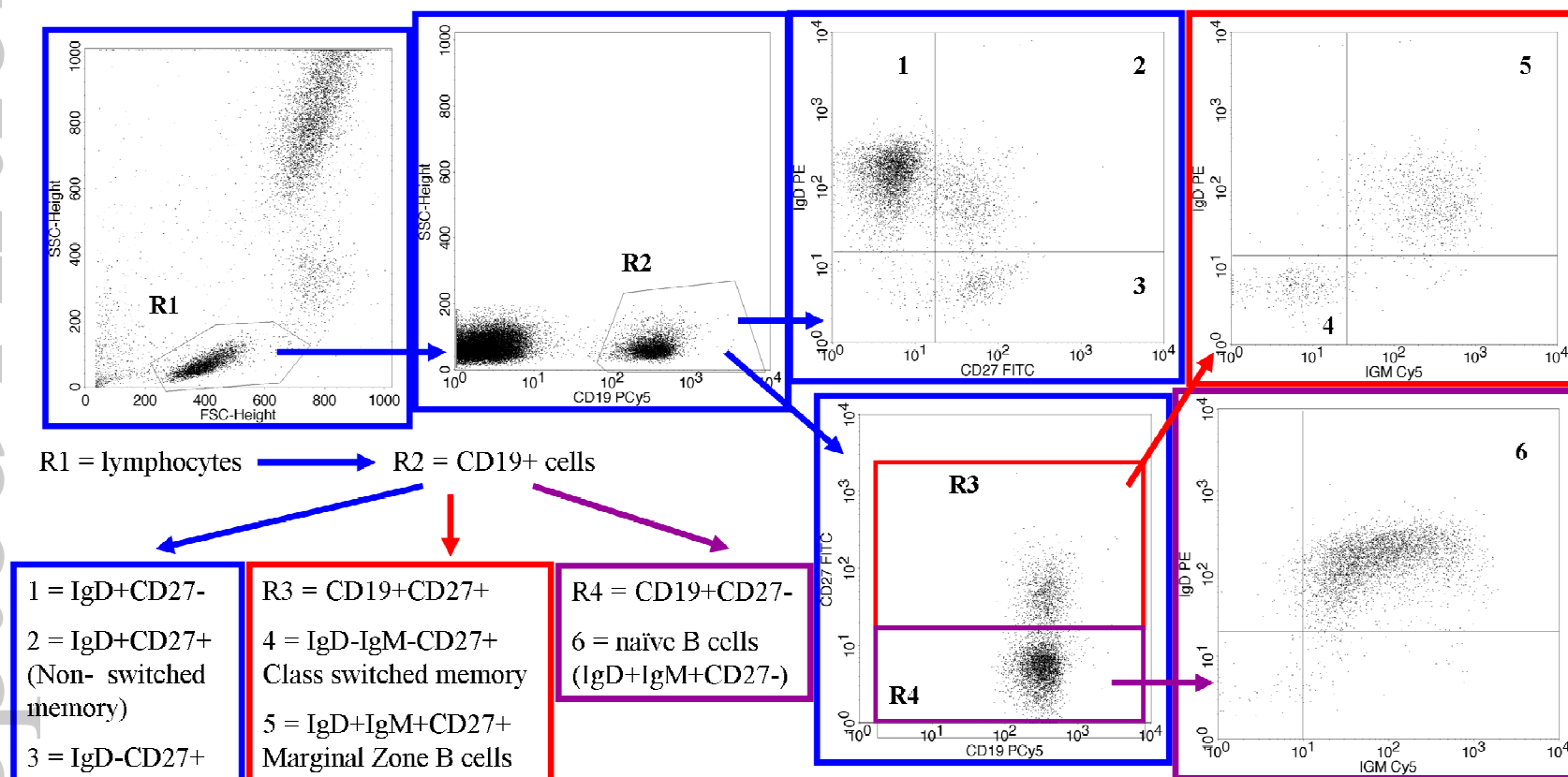


Figure 1 – B-cell gating strategy. The first gate, R1, selects lymphocytes based on side scatter versus forward scatter. These cells are further analysed for CD19 expression and the second gate, R2, selects B-cells (CD19+ cells). These B-cells (R6) are further analysed to determine proportions of non-switched memory, class switched memory, Marginal zone and naïve B-cells.

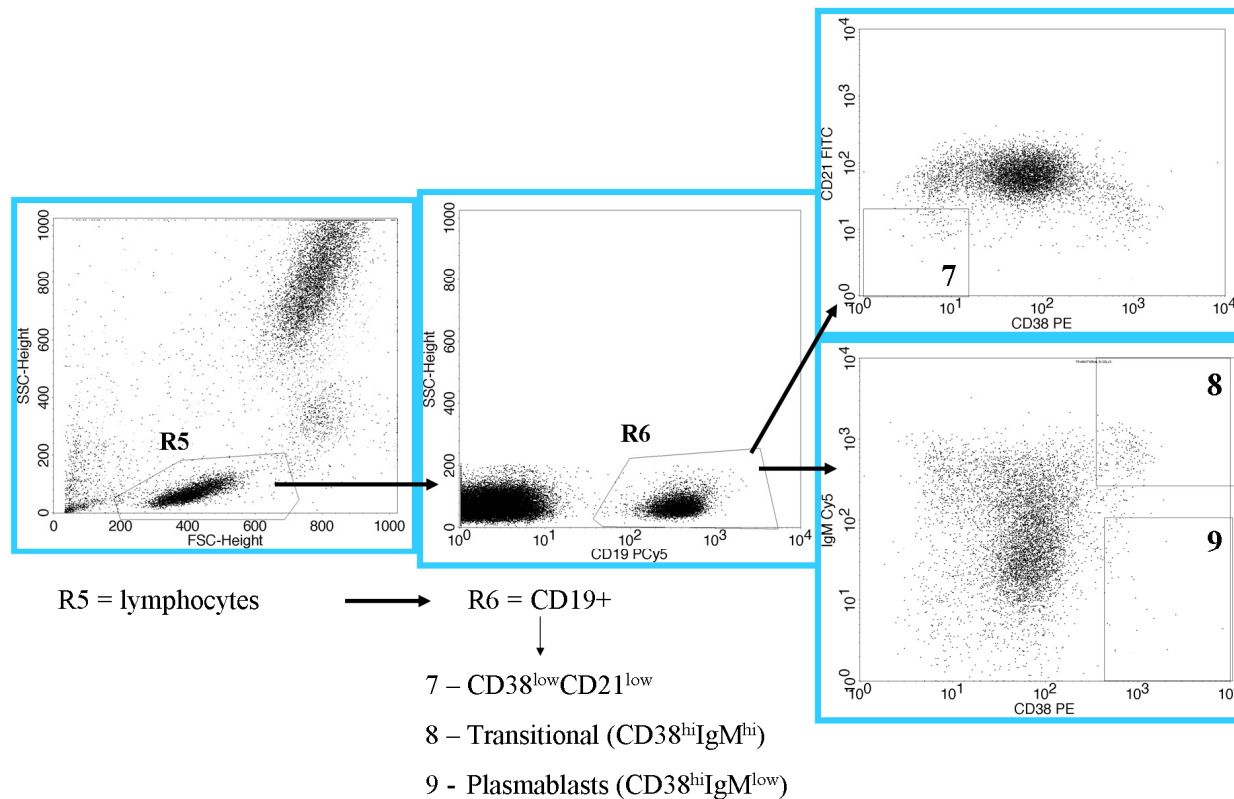


Figure 2 – B-cell gating strategy for determining CD38^{low}CD21^{low}, Transitional B-cells (CD38^{hi}IgM^{hi}) and plasmablast (CD38^{hi}IgM^{low}) subpopulations. The first gate, R5, selects lymphocytes based on side scatter versus forward scatter. CD19 expression is used to select B-cells (CD19+ cells) in the second gate, R6. The B-cells (R6) are further analysed for CD21 and CD38 expression to identify gate 7, CD38^{low}CD21^{low} cells. Alternatively, the B-cells (R6) are analysed for IgM versus CD38 expression to identify gate 8, transitional B-cells (CD38^{hi}IgM^{hi}) and gate 9, plasmablasts (CD38^{hi}IgM^{low}).

A

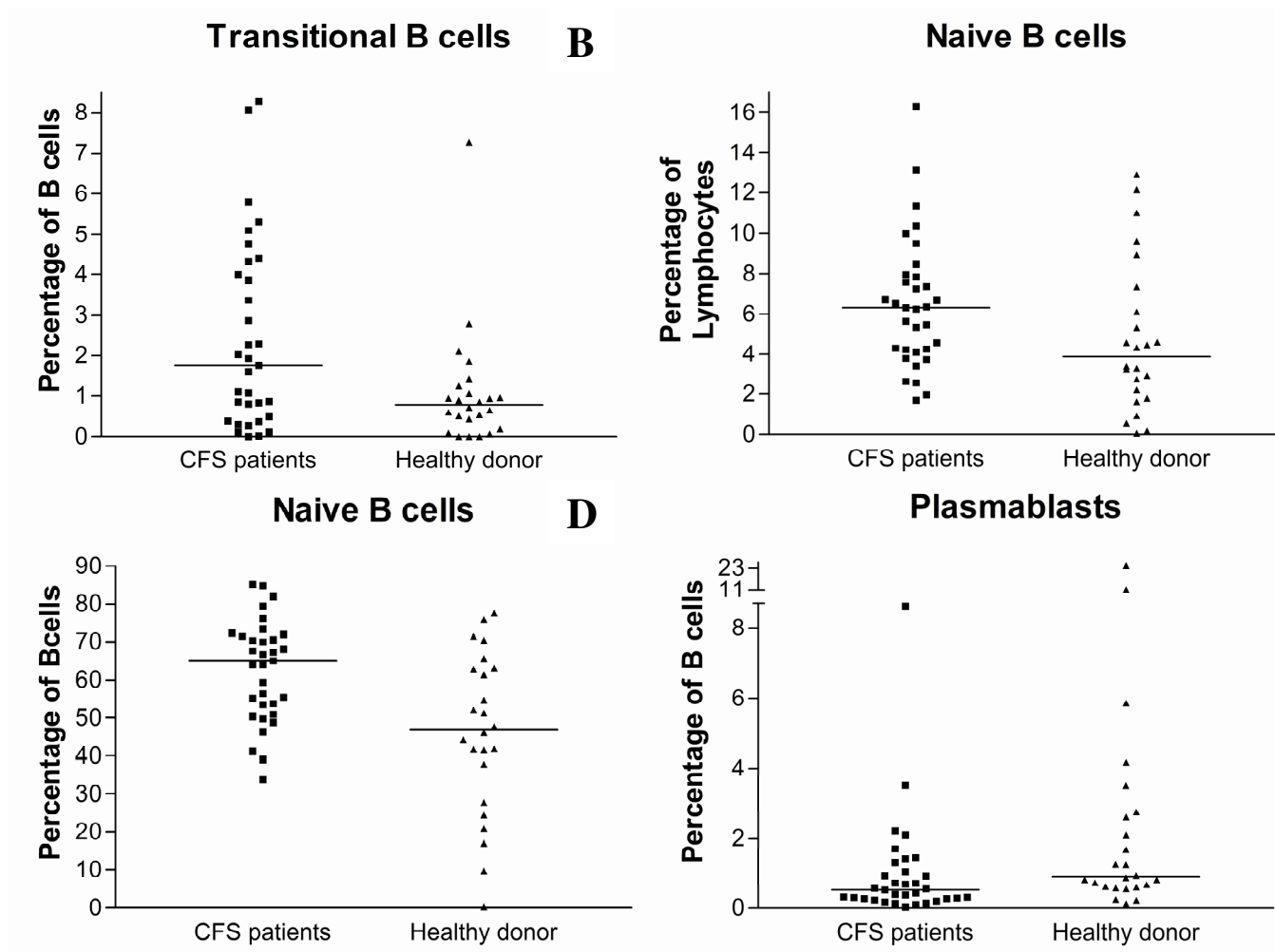


Figure 3 – Graphs summarising B-cell subsets which are significantly different between 33 CFS patients and 24 donors.

A) Percentage of B-cells that are transitional B-cells. CFS patients had 1.8 % versus 0.8 % in controls (P=0.025).

B) Percentage of lymphocytes that are naïve B-cells. CFS patients had 6.3 % versus 3.9 % in HC (P=0.034).

C) Percentage of B-cells that are naïve B-cells. CFS patients had 65 % versus 47 % in controls (P=0.003).

D) Percentage of B-cells that are plasmablasts. CFS patients had 0.5 % versus 0.9 % in controls (P=0.013).