

# Long-Term Cerebrospinal Fluid and Blood Lymphocyte Dynamics After Rituximab for Pediatric Opsoclonus-Myoclonus

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Received: 28 August 2009 / Accepted: 23 September 2009  
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## Abstract

**Introduction** Opsoclonus-myoclonus syndrome (OMS) is an autoimmune paraneoplastic disorder characterized by B and T cell abnormalities in cerebrospinal fluid (CSF) and propensity for relapse. The study aim was to assess whether rituximab-induced B cell ablation in CSF outlasts repopulation in blood and if there are changes in other lymphocyte subsets.

**Materials and Methods** In 25 children with OMS, the expression of CSF and blood lymphocyte surface antigens was evaluated by flow cytometry before and at intervals after rituximab therapy.

**Results** The reduction in CSF CD27<sup>+</sup> memory, CD38<sup>+</sup> activated, CD5<sup>+</sup>, and other B cell subsets was profound ( $p < 0.0001$ ), comparable across groups (−94%), and sustained over 12–18 months despite repopulation in blood. The observed lag in memory B cell pool recovery in the CSF

compared to peripheral blood may be clinically relevant. T cell phenotypic changes involved frequency, not absolute counts, and were transient. Co-treatment with IVIg or ACTH did not significantly alter B cell depletion or repletion.

**Discussion** These data indicate that rituximab affords long-term protection against CSF B cell expansion in OMS (ClinicalTrials.gov NCT00244361).

**Keywords** Anti-B cell therapy · B cell recovery · memory B cells · neuroblastoma · paraneoplastic syndrome

## Introduction

B cell targeted therapy is proving effective in both treating certain autoimmune disorders and clarifying the role of B lymphocytes in their pathogenesis [1]. Rituximab, a first-generation anti-CD20 monoclonal antibody, has been used in a variety of central and peripheral neuroimmunological disorders, from multiple sclerosis [2, 3] to IgM antibody-associated polyneuropathies [4]. In adults, blood B cell reconstitution data exist for some disorders [5, 6], but information on regeneration of cerebrospinal fluid (CSF) B cell subsets is scant. While rituximab is targeted against B cells, recent evidence suggests associated changes in T cells in various lymphoreticular and autoimmune disorders [3, 7]; however, there has been no systematic study.

In children, observations about the effects of B cell depleting agents in the CSF compartment are few. Even in peripheral blood, post-rituximab lymphocyte repopulation has been less well studied than in adults, despite concerns over potential long-term repercussions on the developing immune system. Whether CSF B cell depletion is sustained in childhood neurological disorders characterized by B cell expansion is not known. It is especially

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important to gather this data in relapsing diseases, such as pediatric opsoclonus-myoclonus syndrome (OMS), an immune-mediated paraneoplastic disorder associated with neuroblastoma [8].

This study presents the neuroimmunological data resulting from a phase I/II clinical trial of rituximab for children with OMS, the clinical aspects of which have been reported [9]. Previous work from our group has shown an elevated frequency of B cells in the CSF in OMS [10, 11]. The CSF Th/Ts ratio is reduced, and the frequency of  $\gamma\delta$  T cells may be increased, but there is no information about whether rituximab helps restore the normal phenotype in OMS. In a pilot study at 6 months, rituximab reduced total CSF B cells and was clinically effective when used adjunctively with conventional immunotherapy [12]. The present study is a 12–18-month longitudinal analysis of B and T cell subsets and NK cells in CSF and blood compartments.

**Materials and Methods**

**Study Design**

Children with OMS were recruited from the National Pediatric Myoclonus Center from the USA, Canada, and the UK and examined by the principal investigator to confirm the diagnosis. Parents of 25 patients meeting inclusion and exclusion criteria signed informed consent for this Institutional Review Board approved study (SCRIHS protocol no. 04-112), which was conducted from 2004 to 2007 and registered with the Food and Drug Administration (IND no. 11,771) and ClinicalTrials.gov (NCT00244361). Utilizing methods to obtain CSF atraumatically in children [13], lumbar puncture was done before and at 6 months after completion of rituximab and, in 13 cases, again at 12–18 months (mean 13.8±1.7 SD). CSF was collected for lymphocyte subsets. Blood for lymphocyte subsets was obtained at baseline and at 1, 3, 6, and 12 months.

**Study Drug**

Patients either received rituximab, ACTH, and IVIg at once (Multitherapy Group), rituximab added to already ongoing ACTH and IVIg (Adjunctive Therapy Group), or rituximab alone (Monotherapy Group; Table 1). ACTH and IVIg are considered standard treatments for OMS [14]. Rituximab (Rituxan®), supplied by Genentech, Inc. (South San Francisco, CA)/Biogen IDEC (San Diego, CA), was given IV once weekly for four consecutive weeks at a dose of 375 mg/m<sup>2</sup>. In those treated with ACTH<sub>1–39</sub> (Acthar Gel, 80 IU/mL; Questcor Pharmaceuticals, Union City, CA, USA), a 52-week protocol was initiated at 75 IU/m<sup>2</sup> twice a

**Table 1** Individual B Cell Responses and Case Summaries

Group	Multitherapy										Adjunctive therapy										Monotherapy									
	4	16	12	24	9	14	8	23	11	6	22	13	7	15	21	17	2	10	5	20	1	25	19	3	18					
Case no.	F	M	M	M	M	F	F	F	F	M	M	F	F	F	M	M	F	F	M	F	M	F	F	M	M					
Gender	1.2	2.0	0.9	1.8	1.8	3.0	1.1	1.3	1.5	0.9	1.9	1.4	3.0	1.2	1.6	2.6	3.5	2.4	1.3	0.3	1.5	0.8	1.5	1.3	0.8					
Onset age (years)	0.2	0.2	0.7	0.1	0.3	0.2	0.3	0.1	0.3	0.5	0.1	0.6	5.2	0.4	0.3	0.2	0.2	1.4	0.6	0.8	16	5.4	10	3.3	8.0					
Duration (years)	1.3	2.2	1.6	1.9	2.2	3.2	1.4	1.3	1.8	1.4	2.0	2.0	8.2	1.6	1.9	2.8	3.7	3.8	1.8	1.1	18	6.3	12	4.7	8.8					
Test age	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					
Tumor	25	19	29	30	15	26	22	17	25	23	10	18	11	19	9	10	21	8	17	21	8	14	10	24	4					
TS at base	6	1	8	22	7	2	7	6	6	12	6	5	8	8	4	7	6	3	6	9	6	9	5	19	6					
TS at 6 months	3	3	9	-	9	1	-	2	5	5	6	-	9	3	-	4	-	5	4	4	8	-	4	13	-					
TS at 12 months	3.0	3.8	3.7	5.4	2.7	9.3	4.4	7.4	3.0	3.0	4.8	5.3	2.7	2.7	1.4	4.7	10.5	3.2	3.1	3.5	3.3	2.2	3.4	2.2	2.4					
CSF B 0 month	0.3	1.0	0	-	0	0.1	0	0	0	0.9	0.6	0.4	0.3	1.0	0.1	0	0.2	0	0	0	0	0.4	0	0.5	.01					
CSF B 6 months	0.5	2.9	0.0	-	1.16	0.9	-	-	0.9	-	0.7	1.2	-	-	-	0.1	-	0.4	-	0.8	-	-	0.0	0.2	-					
CSF B 12 months	26	29	24	32	24	20	48	20	28	23	26	15	22	26	32	22	46	18	15	26	18	19	18	26	16					
Bld B 0 month	11	32	0.3	-	20	4	3	8	11	7	17	5	14	24	0.3	0.2	10	27	0	16	0.1	14	0.1	16	11					
Bld B 6 months	18	29	7	-	26	7	-	8	19	-	16	5	24	-	-	4	-	33	10	25	-	-	5	27	-					
Bld B 12 months	0	0	+	0	0	+	+	+	0	0	0	+	0	0	0	0	+	+	0	0	0	0	+	+	+					
Prior relapse	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					
Study relapse	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					

CSF and blood B cell percentages are given by months after completion of rituximab. Patients 2 and 24 were re-evaluated at 3 not 6 months  
TS motor severity total score from 0 to 36

day for 1 week, daily for 1 week, on alternate days for 2 weeks, then slowly tapering to 40 IU/m<sup>2</sup> over 2 months, and more gradually over the next 7 months, until a final dose of 5 IU/m<sup>2</sup> was reached. In IVIg-treated patients, 2 g/kg (divided over 2 days) was used for induction and 1 g/kg once a month with acetaminophen and diphenhydramine pretreatments for maintenance. As intended, the mean time between IVIg infusion and clinical assessments was about 1 month (3.9±1.6 SD weeks).

#### Flow Cytometry of Cerebrospinal Fluid Cells

Fresh CSF and corresponding blood samples were brought for immunophenotyping within 1 h of collection for flow cytometry. The expression of lymphocyte surface antigens was investigated in the CSF and blood using a comprehensive panel of monoclonal antibodies to adhesion proteins in combination with anti-CD3 and anti-CD45 antibodies. Samples were split into several tubes to cover all the stains in the panel. Lymphocytes were stained with directly conjugated monoclonal antibodies to CD5, CD19, CD20, CD27, CD38, CD45, CD3, CD4, CD8, TCR- $\gamma\delta$ , CD16/56, IgD, and Ig isotypes, which were labeled with FITC, PE, APC, or PC5, as described previously [11]. The tube configurations were as follows: 3/4/8/45 (tube 1); 3/16,56/19/45 (tube 2); 4/TCR- $\gamma\delta$ /3 (tube 3); 5/20/19 (tube 4); and IgD/27/38/19 (tube 5). Lymphocyte subsets were defined as follows: B cells (CD45+CD3-CD19+), CD20+ B cells (CD19+CD20+), CD5+ B cells (CD19+CD5+), activated B cells (CD19+CD38+), memory B cells (CD19+CD27+), naive B cells (CD19+CD27-IgD+), T cells (CD45+CD3+), helper/inducer T cells (CD3+CD4+), cytotoxic/suppressor T cells (CD3+CD8+), natural killer-like T cells or NKT (CD3+CD16/56+), gamma/delta T cells (CD3+TCR $\gamma\delta$ +), and natural killer or NK cells (CD3-CD16/56+). Samples were acquired and analyzed on a dual laser FACS caliber flow cytometer (Becton-Dickinson, San Jose, CA, USA) with Cell Quest software (Becton-Dickinson). Quality control was maintained as previously reported. Published reference ranges for blood lymphocytes in healthy children [15] are cited for comparison. Although there are no normative data for CSF lymphocytes in healthy children, data from pediatric neurological controls are available [13]. Rituximab can interfere with the flow cytometric measurement of CD20, but principal B cell comparisons were made predose and at 6 months when rituximab was not detected, and CD19 was our primary marker. “Percent of lymphocytes” denotes percent positive cells.

#### Statistical Procedures

Time-course data were analyzed on the Statistical Analysis System by two-factor analysis of variance (ANOVA) with

repeated measures on one factor, and follow-up comparisons of means were made by the least square means procedure. Results were summarized using descriptive statistics.

## Results

### Blood Immunophenotype

Both the relative size (frequency) and absolute size (cell counts) of blood lymphocyte subsets were evaluated. Because the same patterns were found in all groups without statistically significant differences (Fig. 1a, b), the data were combined for clarity of presentation (Fig. 1c). Treatment with rituximab depleted both relative and absolute blood B cell pools. Blood CD19+ B cells plummeted to 0% by 1 month after the last rituximab infusion, corresponding to a reduction in total B cell counts from 1,095±139 to 1.5±0.3 cells/mm<sup>3</sup> ( $p<0.0001$ ).

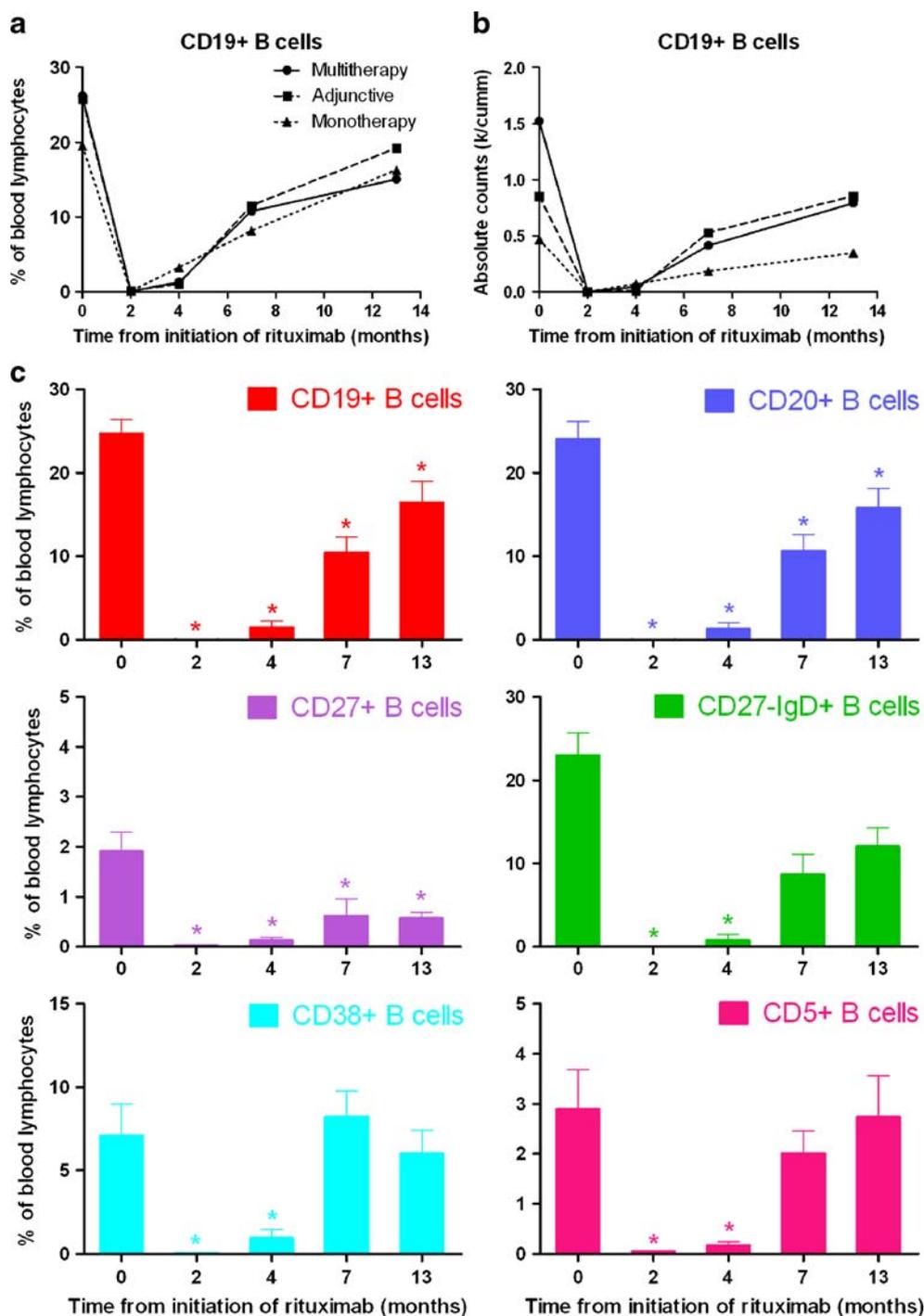
The onset of repopulation was variable, beginning after 1 month following the last rituximab infusion in 18%, after 3 months in 64%, and after 6 months in 18%. The number of subjects with >1% repopulating CD19+ B cells was 18 of 24 at 6 months and 16 of 16 at 12 months. Early repopulation (between 1 and 3 months) occurred in one of 11 in the front-end group, one of seven in the add-on group, and two of four in the monotherapy group. Time to first appearance of B cells was negatively correlated with pretreatment B cell counts ( $r=-0.45$ ,  $p=0.024$ ).

B cell recovery was stepwise and substantial, but the mean frequency at 1 year reached only 67% of the mean baseline ( $p=0.0001$ ) with large individual variation (B cell frequency range 3.8–33%). The median frequency of CD19+ B cells at 1 year (paired data) was 75% of baseline, which also was significantly lower ( $p=0.032$ , Wilcoxon signed ranks test). The B cell count was 754±162 cells/mm<sup>3</sup>, which was 69% of the pretreatment values ( $p=0.015$ ). B cells that were positive for CD20, a marker for mature B cells, showed the same recovery pattern ( $p=0.0004$ ). Compared to the normal reference range, seven subjects were underpopulated (by 1–10%) and two were overpopulated (by 1–2%). Baseline B cell frequencies in OMS fell within the normal reference range, which is wide, however.

B cell subsets responded in like fashion. There was a significant reduction in all B cell subsets ( $p<0.001$ , ANOVA), including CD5+ B cells, CD38+ B cells, CD27+ B cells, and CD27-IgD+ B cells. There was no evidence of disproportional B cell expansion or rebound at repopulation and recovery, except that the frequency of memory B cells remained desirably low.

The percentage of total T cells transiently rose, but drifted back toward pretreatment levels (Fig. 2). There was also an

**Fig. 1** **a** Mean percentage of CD19+ B cells by treatment group (multitherapy,  $p < 0.0001$ ; adjunctive,  $p < 0.0001$ ; monotherapy,  $p = 0.0019$ ). **b** Mean blood B cell counts by treatment group (multitherapy,  $p < 0.0001$ ; adjunctive,  $p = 0.0014$ ; monotherapy,  $p = 0.0019$ ). **c** Longitudinal data for blood B cell subsets in the combined dataset. Data are means  $\pm$  SEM. Asterisks indicate statistically significant differences from pretreatment (time=0) values.  $p < 0.0001$  for all B cells. For reference, the median blood B cell frequency for healthy pediatric controls ages 2–5 years ( $n = 33$ ) is 24% [15]

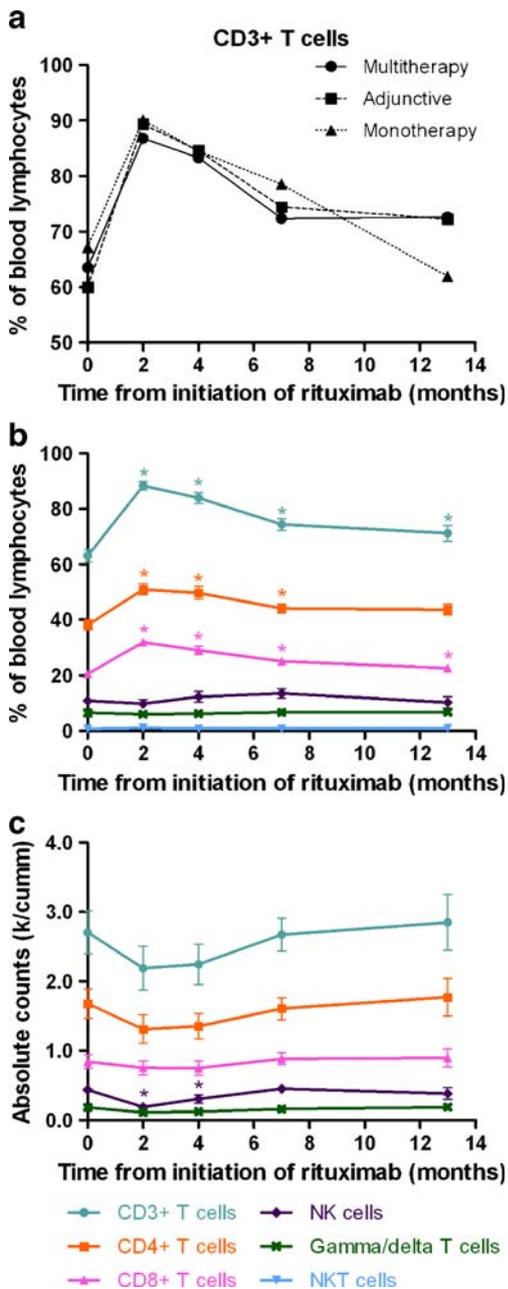


increase in the percentage of CD4+ T cells and CD8+ T cells, with a return to baseline by 1 year. No statistically significant changes in the percentage of blood NK cells or  $\gamma\delta$  T cells were found. Small drops in total T cell and CD4+ T cell counts at 1 and 3 months after the last rituximab were not statistically significant. Decreases in the absolute size of the blood NK cells and  $\gamma\delta$  T cells during that time were

trivial but statistically significant. There were no significant changes in routine complete blood counts.

#### CSF Immunophenotype

At baseline, the frequency of total B cells and B cell subsets (Fig. 3) was elevated approximately 4-fold for CD19+ B



**Fig. 2** **a** Treatment group and mean frequency of blood T cells (all  $p < 0.0001$ ). **b** Longitudinal data for blood T cell subsets and NK cells in combined groups. Data are means  $\pm$  SEM of all data. Asterisks indicate statistically significant differences from pretreatment (time=0) values.  $p < 0.0001$  for total T cells, CD4+ T cells, and CD8+ T cells. For reference, median blood lymphocyte frequencies for healthy pediatric controls ages 2–5 years ( $n=33$ ) are 64% for total T cells, 37% for CD4+ T cells, 24% for CD8+ T cells, and 10% for NK cells [15]. **c** Blood lymphocyte counts

cells and 3-fold for CD20+ B cells (normal  $<1\%$ ). The frequency of total T cells was somewhat reduced, and the CD4/CD8 T cell ratio was low at 1.6 (normal 2–3). The ratio of memory to naive B cell percentage in CSF was

4.8:1; in blood it was 1:12. The frequency of CD5– B cells was substantially higher than that of CD5+ B cells.

In all treatment groups 6 months after the last rituximab infusion, the reduction in frequency of CD19+ CSF B cells was significant: multitherapy ( $p=0.00001$ ), adjunctive therapy ( $p=0.003$ ), and monotherapy ( $p=0.005$ ). CSF B cells were undetectable in 11 of 24 children, a proportion found across groups: five of 11 multitherapy, four of eight adjunctive therapy, and two of five monotherapy. There was a significant reduction in the percentage of all B cell subsets, including CD20+ B cells ( $-94\%$ ), CD5+ B cells ( $-86\%$ ), activated B cells ( $-82\%$ ), memory B cells ( $-96\%$ ), and naive B cells ( $-94\%$ ). No statistically significant difference in depletion among B cell types was found.

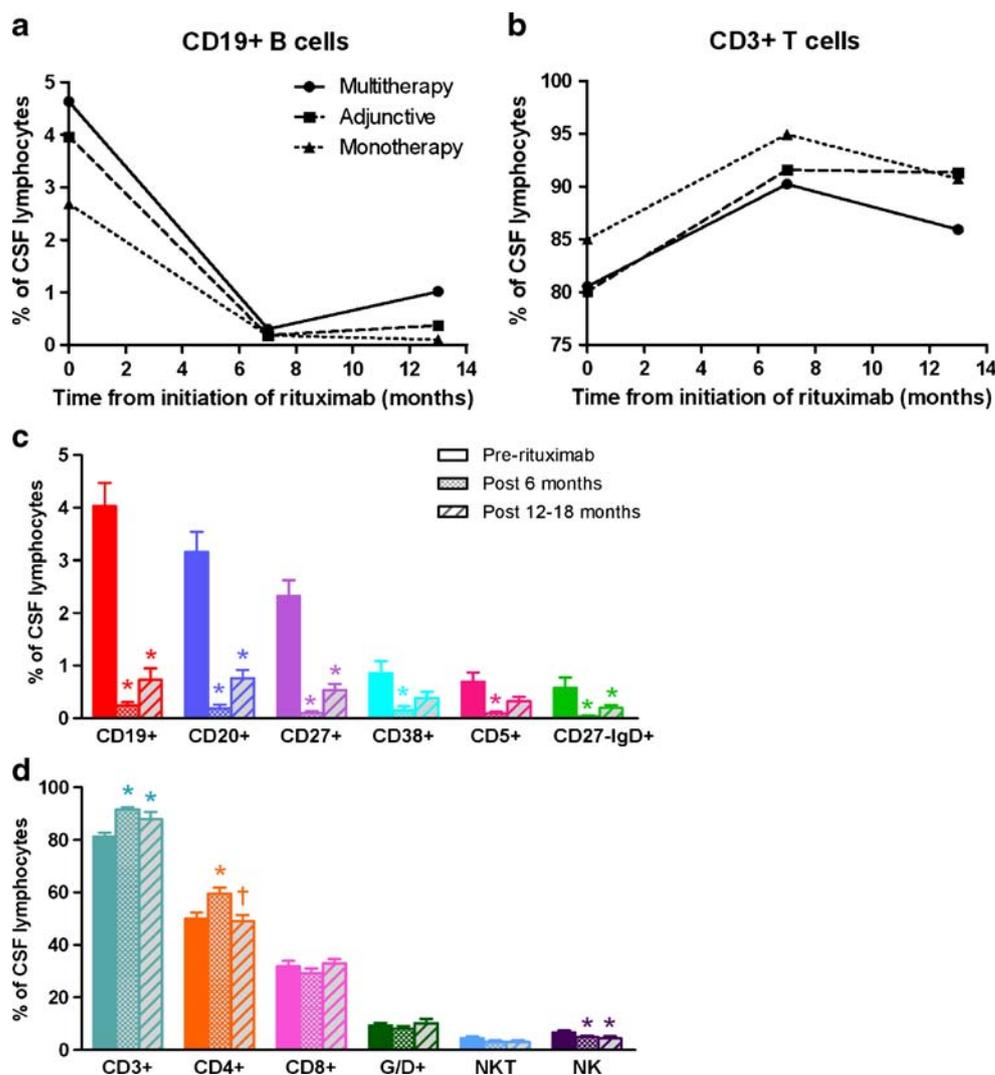
At 12 to 18 months after the final rituximab, B cell frequency remained well below the pretreatment values but slightly higher than the 6 month values, though not significantly so. The mean percentage of CD19+ and CD20+ B cells was still in the normal range ( $<1\%$ ). The memory B cell pool, which had started to recover in the periphery at the time it was depleted in CSF, still lagged behind in CSF compared to peripheral blood.

The frequency of total CSF T cells increased 13%, due to significant changes in multitherapy and adjunctive groups. There also was an 18% rise in the frequency of T-helper/inducer cells. Each of these changes was toward normalization. The frequency of natural killer-like T cells decreased 30%, and that of natural killer cells decreased by 27%. There were no statistically significant changes in the percentage of cytotoxic/suppressor T cells or gamma/delta T cells.

### Discussion

When B cells repopulate in the peripheral blood after anti-B cell therapy, the expectation is that autoreactive B cell clones have been eradicated and that B cells will not overpopulate in the CNS. We have shown previously that total B cells (including CD20+ B cells) do not repopulate in CSF to the same extent as they do in blood 6 months following rituximab therapy [12]. This study extends those findings by demonstrating depletion of various B cell subsets, with low frequency of CSF activated B cells, naive B cells, and memory B cells even 12 to 18 months after treatment. Relative deficiency of memory B cells is ideal; otherwise they migrate to brain as precursors of short-lived plasma cells [16]. Also, the CD5+ B cell subset, which is autoreactive in some autoimmune disorders and expanded in OMS [11], was depleted by rituximab. Without CSF sampling shortly after rituximab treatment, it is not possible to know if the CSF B cells found at 6 months were not ablated or were repopulating.

**Fig. 3** Treatment group and mean frequency of CSF **a** B cells (time effect,  $p < 0.0001$ , no group effect) and **b** T cells (time effect,  $p < 0.0001$ , no group effect). **c** CSF B cell subsets and **d** T cell subsets and NK cells before and 6 or 12–18 months after completion of rituximab infusions. Data are means  $\pm$  SEM. Statistical significance by ANOVA with repeated measures is indicated by *asterisks* for comparisons with pretreatment values and by a *cross* for comparisons between 6 and 12–18 months. For reference, median CSF lymphocyte frequencies for pediatric neurological controls of mean age  $9.3 \pm 1.2$  years ( $n = 16$ ) are 0.71% for total B cells, 0.78% for CD20+ B cells, 0.11% for CD5+ B cells, 86% for total T cells, 68% for CD4+ T cells, 16% for CD8+ T cells, 4.1% for  $\gamma\delta$  T cells, 3.2% for NKT cells, and 6.5% for NK cells (National Pediatric Myoclonus Center database)



We have hypothesized that rituximab depletes CSF B cells by interrupting B cell trafficking into the CNS [12]. Because the instigating tumor is always peripheral in OMS and infiltrated with B cells, it seems reasonable to surmise that, exposed to onconeural antigen, activated B cells penetrate the blood–brain barrier and CSF space. However, low concentrations of rituximab, one-four-hundredth to one-thousandth of serum levels, have been detected in CSF in a small number of adults with multiple sclerosis [17], and whether the concentration is sufficient to contribute to CSF B cell killing is unclear.

The degree and duration of rituximab-induced CSF B cell depletion in OMS is excellent and less variable than that reported for multiple sclerosis treated with the same four-cycle schedule. In two adults with primary progressive multiple sclerosis, rituximab did not deplete CSF B cells, which rebounded several fold by 2 to 18 months [2]. In one adult with fulminant relapsing–remitting multiple sclerosis, CSF B cells were depleted at 2 and 6 months [18], but in another, B cells reappeared in CSF after 5 and 10 months, even before they reappeared in blood [17]. In 10 patients with relapsing–

remitting multiple sclerosis, CSF cells decreased in nine and increased in one, with an overall 90% reduction at 24 weeks [3]. In our study, CSF B cells were depleted in all patients, and only one rebounded into the clearly abnormal range even at 12–18 months. Responsiveness to rituximab is apt to vary by disease and other factors.

In peripheral blood, returning B cells were principally naive, as anticipated for de novo production of B cells, and memory B cells remained low in frequency, as noted in other disorders [19, 20]. IgM, which has a half-life of 10 days, kept falling even after the start of B cell repopulation, whereas IgG and IgA were unaffected. Data like these have suggested that long-lived plasma cells (down-regulated CD20 expression) produce IgG and IgA, whereas short-lived plasma cells or plasmablasts secrete IgM [20]. After shorter-lived plasma cells die off, long-term eradication of memory B cells could result in reduced Ig levels [21].

Are children slower to repopulate circulating B cells than adults? At 12 months after four-dose rituximab for systemic lupus erythematosus in adults, absolute B cell counts had

recovered to about 65% of baseline levels with variability [22]. In rheumatoid arthritis, however, circulating B cell absolute counts returned to baseline 12 months after rituximab [23]. Fifteen months after single-dose rituximab, 78% of adult with kidney allotransplantation and conventional triple immunosuppressive therapy still had profound B cell depletion (<5 cells/mL) [24]. Disease- or trial-related variations may confound a clear comparison with children. It also may be necessary to contrast specific subpopulations of children.

The overwhelming effect of rituximab was on B cells. Given the connectivity of the immune system, however, it was reasonable to hypothesize that compensatory changes occur in other lymphocyte subpopulations in the face of such massive circulating B cell depletion. While there was a higher frequency of total T cells, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells in peripheral blood at 6 months, the effect was transient, and there were no consistent changes in T cell counts. In adults with systemic lupus erythematosus, activated blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells were increased after rituximab [20], and rituximab down-regulated CD40L, CD69, and inducible costimulator on CD4<sup>+</sup> T cells [7]. Lack of change in absolute counts of blood CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells, and NK cells has been reported in patients treated for lymphoma. In adults with multiple sclerosis, T cell number decreased in CSF [3], which may relate to low-level expression of CD20 on a small number of T cells (and NK cells) [25]. In patients with idiopathic thrombocytopenic purpura, pre-rituximab abnormalities of T cells reverted in responders, but were unchanged in nonresponders [26]. Ex vivo T cell function, as monitored by T cell proliferation assays, is not decreased by in vivo rituximab treatment; stimulation indices in the presence of tetanus toxoid increased [27]. It would be interesting to determine whether changes in functionality could be occurring in OMS.

**Acknowledgments** Supported by a clinical trial contract (study number U2743s) to M.R.P. from Genentech, Inc. (South San Francisco, CA)/Biogen IDEC (San Diego, CA) and by grants from the Spastic Paralysis and Allied Diseases of the Central Nervous System Research Foundation (Illinois-Eastern Iowa District Kiwanis International) and Questcor Pharmaceuticals, Inc. (Union City, CA, USA). The authors thank Jennifer A. Swan for dataset graphics, Tammy A. Boyd for typing the manuscript, and patients for providing samples for this analysis.

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