

**Effect of Rituximab on the Peripheral Blood and Cerebrospinal Fluid B cells in Patients with Primary Progressive Multiple Sclerosis**

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## **ABSTRACT**

Context: Rituximab, an anti-CD20 monoclonal antibody that depletes CD20<sup>+</sup> B cells, has demonstrated efficacy in peripheral neurological diseases. Whether this efficacy can be translated to neurological diseases of the central nervous system (CNS) with possible autoimmune B cell involvement remains unknown. Objective: To determine the effect of Rituximab on cerebrospinal fluid (CSF) B cells in Multiple Sclerosis (MS) patients.

Design: Four patients with Primary Progressive MS (PPMS) were treated with Rituximab. CSF and peripheral blood (PB) B cell subsets were identified by flow cytometry from each patient pre- and post-Rituximab. Results: CSF B cells were not as effectively depleted as their peripheral counterparts. Rituximab treatment suppressed the activation state of CSF B cells temporarily. The residual B cells underwent expansion post-Rituximab. Conclusion: The effect(s) of Rituximab on the CSF B cell compartment are limited in comparison to the periphery, but will need to be confirmed in a larger group of MS patients.

## **BACKGROUND**

Multiple Sclerosis (MS) is an inflammatory, demyelinating disease of the Central Nervous System (CNS) which likely involves an autoimmune response directed against myelin associated antigens. Evidence exists that possibly implicate B cells in the development and perpetuation of MS<sup>1-11</sup>. Thus, efforts to address whether B cell depleting therapies may have a place in therapeutic regimens for MS patients have begun.

Rituximab (Genentech, Inc., San Francisco, CA and IDEC Pharmaceuticals, San Diego, CA) is a chimeric murine/human IgG<sub>1</sub> kappa monoclonal antibody. It targets CD20, a transmembrane phosphoprotein expressed on most B cells, except for plasma B cells<sup>12-14</sup>. Rituximab depletes B cells by binding to the CD20 molecule, initiating either complement-dependent cytotoxicity (CDC) or antibody-dependent cell mediated cytotoxicity (ADCC). Rituximab has clear clinical benefit for patients with neoplastic B cell mediated diseases<sup>15</sup> and treatment of anti-GM1 polyneuropathy<sup>16</sup>. Whether this efficacy of Rituximab in peripheral neurological diseases with autoimmune B cell involvement can be translated to neurological diseases of the CNS with possible autoimmune B cell involvement remains unknown.

## **OBJECTIVE**

The objective of this study was to determine the effect of Rituximab on B cells in the PB and CSF of MS patients.

## **METHODS**

Study Population: Five patients were recruited for Rituximab therapy at the University of Texas Southwestern Medical Center at Dallas based on prior response to plasmapheresis and continued disease progression. All patients signed a consent form approved by the IRB. Their clinical presentation is summarized in Table 1.

Therapy: All five patients were treated with Rituximab at a dose of 375 mg/m<sup>2</sup> IV weekly for 4 consecutive weeks.

B cell frequency assessment: Four of the five patients consented to donate a PB sample prior to, and after completion of Rituximab therapy. Approximately 10 cc's of

heparinized PB was collected at each sampling. Mononuclear cells from the PB were isolated by ficoll-hypaque sedimentation as described<sup>17</sup>. Two of these patients also consented to donate CSF at the same time points as the blood samplings. CSF cells were isolated by centrifugation of the CSF. PB and CSF cells were stained with a PE-labeled anti-CD19 mAb and other markers that identify B cell subsets (Table 2). The cells were collected using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA), and the CD19<sup>+</sup> B cell frequencies and light scatter characteristics analyzed using Cellquest software (Becton Dickinson, San Jose, CA). CD19<sup>+</sup> B cell frequencies (pre- and post-Rituximab) in the PB and CSF were compared using the  $\chi^2$  test.

## RESULTS

***Effect of Rituximab on Peripheral B cell Frequency.*** Both PB and CSF were collected from two MS patients (M116 and M154) pre- and post-Rituximab. Only PB was sampled from MS patient M602 and one patient with progressive myelopathy (M191). The samples were stained for CD19, a B cell marker whose expression parallels that of CD20<sup>18</sup>.

CD19<sup>+</sup> B cells are effectively depleted in the periphery following Rituximab therapy in all four patients (Table 3), as expected. M116 had 18.43% CD19<sup>+</sup> PB B cells pre-Rituximab, which decreased to 0.14% 1 month post-Rituximab (Table 3). The other three patients demonstrated similar significant decreases in CD19<sup>+</sup> PB B cell frequencies post-Rituximab. Of note, M116 had a second course of Rituximab, and two months after this second course, the CD19<sup>+</sup> B cell population was not depleted (compare 4.42% to 7.91%). This resistance to depletion upon re-treatment with Rituximab has been reported<sup>19</sup>.

The time frame in which CD19<sup>+</sup> PB B cell frequencies recovered varied between the patients (Table 3). The CD19<sup>+</sup> B cell frequency in M116 had increased to 4.42% 18 months post-Rituximab, but did not fully recover to the pre-Rituximab B cell frequency of 18.43%. M191 also did not recover to the pre-Rituximab B cell frequency at 14 months post-Rituximab. In contrast, M154 had a larger frequency than observed pre-

Rituximab. Thus, there will likely be individual variability in the time frame and extent of CD19<sup>+</sup> PB B cell frequency recovery post-Rituximab.

***Effect of Rituximab on B cell Frequency in the CSF.***

Only one of the patients demonstrated a significant decrease in CD19<sup>+</sup> CSF B cell frequency post-Rituximab (M116, 1.03% pre-Rituximab vs 0.63% post-Rituximab,  $p=0.0001$ )(Table 4). In contrast, the CD19<sup>+</sup> CSF B cell frequency in the CSF of M154 post-Rituximab did not decrease (1.74% pre-Rituximab vs 3.44% post-Rituximab,  $p=0.141$ ). Thus, depletion of CSF B cells post- Rituximab cannot be achieved as consistently as in the periphery.

The CD19<sup>+</sup> B cell frequency in M116 18 and 20 months post-Rituximab was significantly greater than the pre-Rituximab sampling, whereas the CD19<sup>+</sup> B cell frequency of M154 14 months post-Rituximab remained the same as the earlier post-Rituximab sampling (Table 4). Thus, recovery of the CD19<sup>+</sup> CSF B cell frequencies post-Rituximab is likely to vary among individuals. In order to provide an accurate time frame in which Rituximab depletes B cells most effectively in the CSF, a thorough kinetic analysis of B cell frequencies in a larger patient population would be required.

***Analysis of the Residual B cell population in M116.*** There was no decrease in the CD19<sup>+</sup> B cell frequency two months after M116 had received a second course of Rituximab (Tables 3 and 4). In order to discern what B cell subtypes survived the second Rituximab course, we stained the PB and CSF with CD19, CD38, IgM and IgD. Recently activated B cells express CD19/CD20 very brightly and are CD38<sup>-</sup>IgM<sup>+</sup>IgD<sup>-</sup>, whereas advanced memory (post-switch and pre-plasma B cells) dimly express CD19/CD20 and are CD38<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup> (see Table 2).

The CD19<sup>+</sup> PB B cell population of M116 could be subdivided into two separate populations: CD19<sup>bright</sup> and CD19<sup>dim</sup>. The majority of the CD19<sup>+</sup> B cells that remained post-Rituximab was CD19<sup>dim</sup> (87%)(Figure 1A), IgM<sup>-</sup>IgD<sup>-</sup> (Figure 1B), and did not express CD38 (see rectangular gate in Figure 1C). Thus, the residual B cells were post-switch B cells that had not yet differentiated to a plasma B cell phenotype. There was also a population of cells that were CD19<sup>bright</sup>CD38<sup>+</sup>IgM<sup>+</sup>IgD<sup>+</sup> (see oval gate in Figure 1C), which are likely immature B cells (see Table 1) that have recently emerged from the bone marrow to begin repopulating the peripheral B cell pool.

Approximately 80% of the CD19<sup>dim</sup> CSF B cells were IgM<sup>-</sup>IgD<sup>-</sup>CD38<sup>+</sup> (see Figure 1E and rectangular gate in Figure 1F), which stratified them as plasma B cells. The other 20% of the CD19<sup>dim</sup> B cells were IgM<sup>-</sup>IgD<sup>-</sup>CD38<sup>-</sup>, and had thus not differentiated to a plasma B cell phenotype. The CD19<sup>-</sup>CD38<sup>+</sup> population would include plasma B cells as well, but this population also contains T cells, so an accurate analysis of the plasma B cells in this population could not be ascertained.

***Activation Status of Peripheral B cells in response to Rituximab*** Activated B cells (such as plasma B cells) are generally larger and more granular than their naïve counterparts<sup>20-23</sup>. This phenomenon can be observed by flow cytometric light scatter characteristics, looking at the placement of B cells in the FSC/SSC plot, which separates cells by size and granularity respectively. To identify B cell subpopulations post- Rituximab, we re-analyzed the B cell compartments in the CSF and PB from the pre- and two post- Rituximab time points of M116 and M154.

There were two populations of CD19<sup>+</sup> PB B cells (bright and dim) in M116 (Figure 1A), so we analyzed them separately for their light scatter profiles. The majority of B cells in the PB of M116 pre-Rituximab were CD19<sup>bright</sup> (78.8%) (Figure 1A), and were mostly contained in the typical lymphocyte gate (i.e. low FSC/SSC) (red dots) (Figure 2A). The CD19<sup>dim</sup> B cells (green dots) (Figure 2A) were also mostly contained in the typical lymphocyte gate (i.e. low FSC/SSC). This indicated that the CD19<sup>bright</sup> and CD19<sup>dim</sup> B cells were largely in a resting state.

One month post-Rituximab, the CD19<sup>bright</sup> B cells (red dots) were largely depleted (Figure 2B), as expected. However, the CD19<sup>dim</sup> B cells (green dots) remained, and maintained their resting state (low FSC/SSC). Eighteen months later, the CD19<sup>bright</sup> B cells (red dots) had repopulated the B cell pool (Figure 2C), and were in a resting state as had been observed pre-Rituximab. Interestingly, 18 months post-Rituximab, the CD19<sup>dim</sup> B cell population (green dots) had not only expanded (3.9% of CD19<sup>+</sup> B cells pre-Rituximab vs 96.4% of CD19<sup>+</sup> B cells post-Rituximab), but had increased their forward scatter (i.e. were larger in size) to a position above the lymphocyte region (Figure 2C). This light scatter profile is consistent with a more activated state of the CD19<sup>dim</sup> B cells than observed pre-Rituximab.

The peripheral B cells from M154 behaved in a similar manner (Figure 3), with the CD19<sup>bright</sup> B cells (red dots) located in the typical lymphocyte region pre-Rituximab (Figure 3B), followed by depletion of the CD19<sup>bright</sup> B cells 2 months post-Rituximab (Figure 3C), and finally, repopulation of the typical lymphocyte region 14 months post-Rituximab with CD19<sup>bright</sup> B cells (Figure 3D). The CD19<sup>dim</sup> B cells (green dots) were also present pre-Rituximab (Figure 3B), but remained largely in the typical lymphocyte region (low FSC/SSC), and remained so 2 months post-Rituximab (Figure 3C). Fourteen months later, the CD19<sup>dim</sup> B cell population had expanded (20.1% of CD19<sup>+</sup> B cells pre-Rituximab vs 91.9% of CD19<sup>+</sup> B cells post-Rituximab), and were in a more activated state.

#### ***Activation Status of CSF B cells in response to Rituximab***

We have observed that the majority of CSF B cells are not located within the typical lymphocyte region, but instead have increased light scatter profiles to reflect a more activated phenotype. The CSF B cells from M116 are a typical example of that, having few CD19<sup>bright</sup> B cells (red dots) relative to PB (compare Figure 2A to 2D), and the majority of CD19<sup>dim</sup> B cells (green dots) located above the resting lymphocyte region (Figure 2D) pre-Rituximab. Interestingly, one month post-Rituximab, the majority of CSF B cells were CD19<sup>dim</sup> (green dots), and had re-located to the resting lymphocyte region (Figure 2E). Twenty months post-Rituximab, some CD19<sup>bright</sup> CSF B cells (red dots) were observed within the typical lymphocyte region. However, the CD19<sup>dim</sup> B cells (green dots) had increased light scatter profiles, indicating a heightened state of activation (Figure 2F), similar to their light scatter profile pre-Rituximab. In addition, the CD19<sup>dim</sup> B cells (green dots) had expanded in comparison to the pre-Rituximab population (55% of CD19<sup>+</sup> B cells pre-Rituximab vs 77% CD19<sup>+</sup> B cells post-Rituximab).

The majority of CSF B cells from M154 pre-Rituximab was mostly CD19<sup>dim</sup> (77.8%), and had a high light scatter profile (Figure 3F). We had observed the same profile of CSF B cells from M116, and consider this pattern typical for CSF B cells, which are more activated than their peripheral counterparts. Two months post-Rituximab, some CD19<sup>bright</sup> CSF B cells (red dots) were still detectable (Figure 3G), but the CD19<sup>dim</sup> B cells (green dots) remained, and interestingly, displayed a low light scatter profile indicative of a resting state (Figure 3G). However, 14 months post-Rituximab,

some CD19<sup>bright</sup> CSF B cells (red dots) had repopulated. The CD19<sup>dim</sup> B cells (green dots) had expanded significantly (20.2% of CD19<sup>+</sup> B cells pre-Rituximab vs 94.4% of CD19<sup>+</sup> B cells post-Rituximab) and had assumed their activated state (high light scatter)(compare Figure 3H to 3F).

## DISCUSSION

It is evident that CD19<sup>+</sup> B cells were depleted in the periphery within 1-2 months post-Rituximab in the PB of all three patients. Even M191 maintained a depleted CD19<sup>+</sup> B cell population in the periphery 14 months post-Rituximab. However, the PB CD19<sup>+</sup> B cell population was either partially or fully reconstituted between 14 and 18 months post-Rituximab, indicating that the inhibitory effect of Rituximab on B cells no longer exists.

The effect of Rituximab on the B cell pool within the CNS tissue is not as well-defined, but becomes an issue when considering Rituximab as a therapeutic agent in B cell mediated diseases of the CNS. We have examined the effect of Rituximab on CD19<sup>+</sup> CSF B cells in two MS patients, which likely represents a subset of B cells that populate the CNS tissue. The CD19<sup>+</sup> CSF B cells showed a mild decrease 1 month post-Rituximab, followed by a significant expansion 18 and 20 months post-Rituximab that was 5-fold and 9-fold the pre-Rituximab CD19<sup>+</sup> B cell frequency. However, the CD19<sup>+</sup> CSF B cells in M154 were not affected by Rituximab. This was in direct contrast to what had been routinely observed in PB B cells in response to Rituximab, and prompts the question of whether this lack of CSF B cell depletion has any repercussions on the potential efficacy of Rituximab treatment of MS.

There are at least two possible scenarios that could account for the lack of CSF B cell depletion. First, the concentration of Rituximab that reaches the CSF can be nearly 10-fold less than the concentration readily achieved in the PB<sup>24</sup>. Thus, if therapeutic levels of Rituximab cannot reach the CSF, depletion of CSF B cells should not be expected. However, the observation that only 10% or less of the Rituximab concentration that is reached in the PB is obtainable in the CSF is based on patients whose blood brain barrier (BBB) is not compromised as it is in most MS patients. Thus, perhaps higher CSF Rituximab concentrations can be achieved in MS patients whose BBB has been compromised. This concept has not been rigorously tested. Second,

Rituximab is more effective at depleting B cells that have high expression of CD20—such as naïve B cells. However, most CSF B cells are advanced memory or plasma B cells, which express CD19/20<sup>18</sup> dimly, rendering them resistant to Rituximab.

Rituximab also appears to affect CD19<sup>dim</sup> B cell activation states since the initial activated phenotype of the CD19<sup>dim</sup> CSF B cells was suppressed post-Rituximab. However, these CD19<sup>dim</sup> CSF B cells had re-established their activation phenotype 14 and 20 months post-Rituximab. It is unclear how the suppression of this activation state shortly after Rituximab therapy occurs. It is possible that Rituximab itself asserts some form of suppression on residual B cells, or that some other changes in the CSF environment in response to Rituximab causes CD19<sup>dim</sup> B cells to enter a resting state. What affect this resting state of the residual CD19<sup>dim</sup> CSF B cells has on disease status remains unknown, although it is compelling to predict that this suppression of the residual B cells by Rituximab (directly or indirectly) will translate to a clinical improvement for the patients. However, it is clear that at some point (presumably when Rituximab levels drop below a certain concentration), the surviving CSF B cells resume their activated phenotype.

This activation suppression phenomenon we had observed with the CSF CD19<sup>dim</sup> B cells was not observed in the peripheral CD19<sup>dim</sup> B cell population because these cells are typically resting. However, the CD19<sup>dim</sup> B cells that survived in the periphery had acquired an activated state post-Rituximab. Whether this activation state of CD19<sup>dim</sup> peripheral B cells parallels that of the CD19<sup>dim</sup> CSF B cells will need to be investigated, as well as what effect this might have on the reconstituted B cell pool.

We also observed an expansion of PB and CSF CD19<sup>dim</sup> B cells post-Rituximab. Expansion of residual CD19<sup>dim</sup> B cells may be based on the theory of space threshold which suggests that there is a finite amount of space which cells are allowed to populate, and once the CD19<sup>bright</sup> B cells were depleted, the CD19<sup>dim</sup> B cells took advantage of the extra space, and expand as observed in these two patients. Alternatively, the chemokine milieu may have shifted during Rituximab therapy, resulting in an influx of CD19<sup>dim</sup> B cells from other compartments (such as the bone marrow), and subsequent expansion. Third, perhaps residual CD19<sup>bright</sup> B cells that survived Rituximab treatment were able to rapidly become activated and differentiate. However, in order for this hypothesis to

apply to the CSF B cells, the concept that B cells can differentiate and become activated outside the context of germinal centers must be substantiated.

In summary, we have observed typical depletion of PB B cells post-Rituximab in PPMS patients. Rituximab does not deplete CSF B cells efficiently because most CSF B cells are highly activated advanced memory or plasma B cells that express low or no CD20. Recovery of the initial B cell frequencies will likely vary among individuals. Alterations in activation and expansion status of PB and CSF B cells in response to Rituximab requires further study to resolve the mechanism(s) underlying these phenomena.

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**Table 1: Patient Summary<sup>A</sup>**

	Age/Sex	Type of MS	Time Since Initial Symptoms	Time Since Diagnosis	EDSS (pre-RIT)	MRI Findings	OCB	IgG Synthesis <sup>B</sup>
M116	61/F	PP <sup>C</sup>	23 yrs	20 yrs	7	Periventricular T2 lesions	Negative	8.36
M154	59/F	PP	26 yrs	22 yrs	6	Periventricular T2 lesions	Positive	0.85
M188	54/F	PP	5 yrs	3 yrs	8	Periventricular T2 lesions	Negative	0.00
M191	52/F	OND	2 yrs	1 yr	ND	Normal	Negative	ND
M602	44/M	PP	24 yrs	17 yrs	7	Cervical Atrophy	ND	ND

<sup>A</sup> Abbreviations used in this table: Multiple Sclerosis (MS), Expanded Disability Status Scale (EDSS), pre-Rituximab (pre-RIT), Magnetic Resonance Imaging (MRI), Oligoclonal Banding (OCB), Primary Progressive (PP), Other Neurological Disease (OND)

<sup>B</sup> The normal range for IgG synthesis is between 0 and 12

<sup>C</sup> This patient also had elevated anti-GM1 and anti-sulfatide antibodies and a co-existent peripheral neuropathy

**Table 2: Marker Expression on B cell subsets**

	<b>CD19/20</b>	<b>FSC/SSC</b>	<b>CD38</b>	<b>IgM</b>	<b>IgD</b>
Immature	Bright	Low	<b>+</b>	<b>+</b>	<b>+</b>
Naive	Bright	Low	-	<b>+</b>	<b>+</b>
Recently Activated	Bright	Low	-	<b>+</b>	-
Advanced Memory <sup>A</sup>	Dim	High	-	-	-
Plasma B cells	Dim/-	High	<b>+</b>	-	-

<sup>A</sup> Includes post-switch memory and pre-plasma B cells

**Table 3: CD19+ Frequencies in the Peripheral Blood of MS Patients Receiving Rituximab**

Patient Code	Time of Sampling	CD19 <sup>+</sup> in PBL
M116	Pre-Rituximab	18.43%
	1 month Post Rituximab	0.14% <sup>B</sup>
	18 months Post Rituximab <sup>A</sup>	4.42% <sup>B</sup>
	20 months Post Rituximab	7.91% <sup>B</sup>
M154	Pre-Rituximab	3.80%
	2 months Post Rituximab	0.02% <sup>B</sup>
	14 months Post Rituximab	7.42% <sup>B</sup>
M191	Pre-Rituximab	6.3%
	14 months Post Rituximab	1.58% <sup>B</sup>
M602	Pre-Rituximab	6.5%
	2 months Post Rituximab	0.32% <sup>B</sup>

<sup>A</sup> M116 was re-treated with Rituximab immediately following this sampling

<sup>B</sup> Statistically different from the Pre-Rituximab CD19+ frequency in the PB, according to chi-square analysis

**Table 4: CD19<sup>+</sup> Frequencies in the CSF of MS Patients Receiving Rituximab**

Patient Code	Time of Sampling	CD19 <sup>+</sup> in CSF
M116	Pre-Rituximab	1.03%
	1 month Post Rituximab	0.63% <sup>B</sup>
	18 months Post Rituximab <sup>A</sup>	5.63% <sup>B</sup>
	20 months Post Rituximab	9.24% <sup>B</sup>
M154	Pre-Rituximab	1.74%
	2 months Post Rituximab	3.44% <sup>C</sup>
	14 months Post Rituximab	3.31% <sup>C</sup>

<sup>A</sup> M116 was re-treated with Rituximab immediately following this sampling

<sup>B</sup> Statistically different from the Pre-Rituximab CD19<sup>+</sup> frequency in the CSF, according to chi-square analysis

<sup>C</sup> Not statistically different from the Pre-Rituximab CD19<sup>+</sup> frequency in the CSF, according to chi-square analysis

## FIGURE LEGENDS

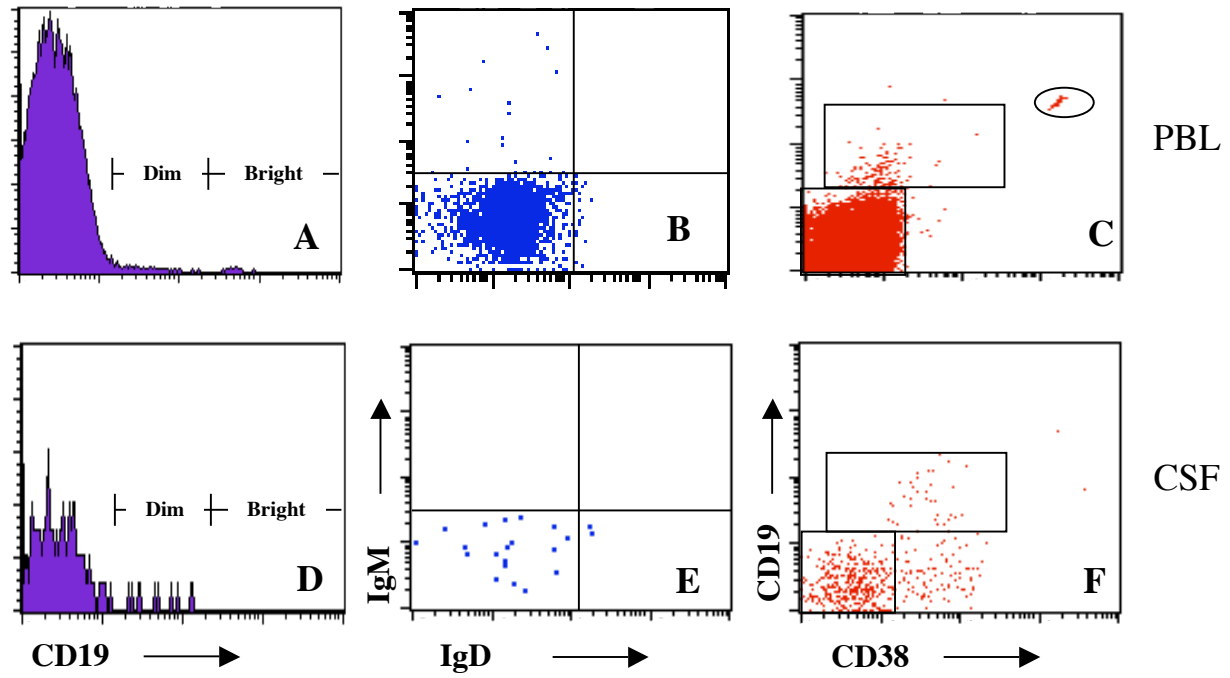
Figure 1. B cell representation in M116 after Re-treatment with Rituximab. Peripheral blood (Panels A-C) and CSF (Panels D-F) B cells were stained for CD19, IgM, IgD, and CD38. Panel designations (A-F) are indicated in the bottom right hand corner of each panel. Panels A and D are histograms, demonstrating the position of CD19<sup>dim</sup> and CD19<sup>bright</sup> populations within the lymphocyte population. Panels B and E illustrate the IgM and IgD expression of the CD19<sup>+</sup> B cells. Panels C and F illustrate the co-expression of CD38 on the CD19<sup>+</sup> B cells. The rectangular gate indicates the CD19<sup>dim</sup> B cell population discussed in the text. The oval gate in Panel C indicates the CD19<sup>bright</sup>CD38<sup>bright</sup> population. The square gate in Panels C and F indicate cells negative for both CD19 and CD38.

Figure 2. B cell activation states in the PB and CSF of M116. Panel designations (A-F) are indicated in the bottom right hand corner of each panel. PB (Panels A-C) and CSF (Panels D-F) were stained for CD19. Red dots indicate the location of CD19<sup>bright</sup> B cells, whereas green dots indicate the location of CD19<sup>dim</sup> B cells in Panels A-C and Panels D-F. Panels A and D indicate the Forward/side scatter designations for the PB and CSF cells prior to receiving Rituximab. Panels B and E indicate the Forward/side scatter designations for the PB and CSF cells 1 month post-Rituximab. Panels C and F indicate the Forward/side scatter designations for the PB and CSF cells 18 months post-Rituximab. The y-axis scale for Panel F is linear, rather than log.

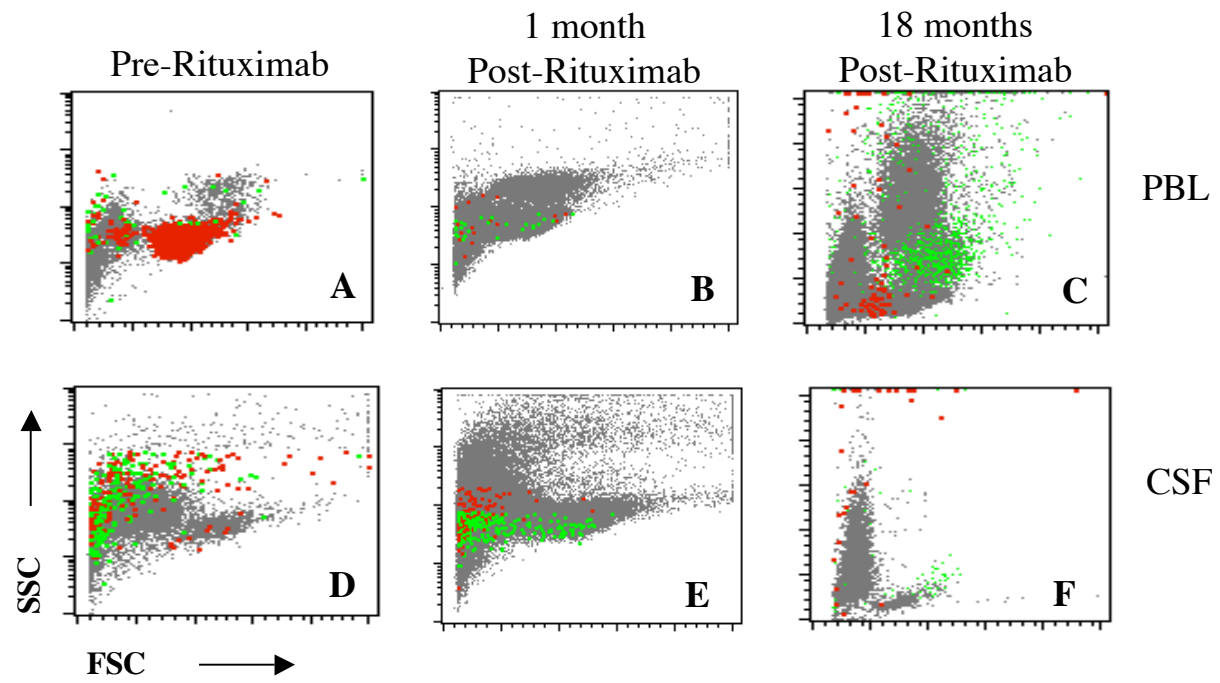
Figure 3. B cell activation states in the PB and CSF of M154. Panel designations (A-H) are indicated in the bottom right hand corner of each panel. PB (Panels A-D) and CSF (Panels E-H) were stained for CD19. Red dots indicate the location of CD19<sup>bright</sup> B cells, whereas green dots indicate the location of CD19<sup>dim</sup> B cells in Panels B-D and Panels F-H. Panels A and E are histograms indicating the CD19<sup>dim</sup> and CD19<sup>bright</sup> designations. Panels B and F indicate the Forward/side scatter designations for the PB and CSF cells prior to receiving Rituximab. Panels C and G indicate the Forward/side scatter designations for the PB and CSF cells 2 months post-Rituximab. Panels D and H indicate

the Forward/side scatter designations for the PB and CSF cells 14 months post-Rituximab. The y-axis scale for Panels D and H is linear, rather than log.

**FIGURE 1**



**FIGURE 2**



**FIGURE 3**

