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B cell depletion with ublituximab reshapes the T cell profile in multiple sclerosis patients

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ABSTRACT

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system, thought to be mediated by myelin-specific CD4+ T cells. However, B cell depletion has proven to be an effective therapy for MS, but the mechanism is not well understood. This study was designed to determine how B cell depletion changes lymphocyte profiles. During a phase IIa clinical trial with ublituximab, a novel CD20 antibody, blood was collected from 48 MS patients at 11 time points over 24 weeks and the lymphocyte profiles were analyzed by flow cytometry. The percentage of naïve CD4+ and CD8+ T cells increased, while the percentage of both effector and central memory T cells declined. CD4+ Th1 effector cells decreased, while there was a significant increase in CD4+ regulatory T cells. The depletion of B cells had a favorable shift in the lymphocyte landscape, reducing the number of naïve T cells becoming activated and transitioning to memory T cells. The ratio of Th1 cells to CD4+ regulatory T cells declined, suggesting that immune regulation was being restored. These data suggest that loss of B cells as antigen presenting cells is a major mechanism of action for the beneficial effects of CD20 antibody therapy in MS.

1. Introduction

B cell depletion has proven to be beneficial in the treatment of relapsing-remitting and primary progressive multiple sclerosis (MS; Hauser et al., 2008; Bar-Or et al., 2008; Hawker et al., 2009; Kappos et al., 2011; Montalban et al., 2017; Hauser et al., 2017); however, the mechanism of action has yet to be elucidated. Intrathecal production of antibodies, observed as the hallmark oligoclonal bands in the CSF, was suggestive that antibodies may be contributing to disease pathology (Li et al., 2018). Strategies to deplete antibodies from MS patients have had limited clinical benefits (Gordon et al., 1985; Weinshenker et al., 1999; Fazekas et al., 2005; Pohlau et al., 2007; Fazekas et al., 2008), suggesting that auto-reactive antibodies are not a major contributor to disease pathology in most MS patients. Anti-CD20 monoclonal antibody therapy is used to deplete B cells in MS patients, resulting in > 99% loss of peripheral blood B cells. Since CD20 is not expressed on plasmablasts or plasma cells, the antibody secreting B cells are not depleted, and therapeutic benefits are observed without changes in antibody titers. Thus, antibody-independent mechanisms are likely responsible for the clinical benefit observed with B cell depletion therapy, as previously recognized with no change in CSF IgG index, IgG concentration, IgG synthesis rate, or oligoclonal bands in MS patients treated with rituximab accompanied by an 88% reduction in gadolinium-enhancing lesions (Cross et al., 2006; Naismith et al., 2010).

B cells play two other major roles in immune responses. B cells are antigen presenting cells (APCs) and modulate T cell responses. Their unique ability to bind specific conformational antigens via their B cell receptor allows them to be very efficient APCs of low abundance

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Table I
Inclusion and exclusion criteria for study enrollment.

Inclusion criteria	Exclusion criteria

• 18–55 age
• Diagnosis of RMS (McDonald criteria 2010)
  • ≥ 2 relapses in prior 2 years or 1 relapse in the past year and/or ≥ 1 Gd enhancing lesion
• Active disease
• EDSS 0–5.5
• B cell counts ≥5% of total lymphocytes
  • Neurologic stability ≥30 days prior to screening and baseline
• Female subjects who are not of child-bearing potential, and negative serum pregnancy test
• Willingness and ability to comply with trial and follow-up procedures, and give written consent
	• Treatment with anti-CD20 or other B cell agent in last 12 mo
	• Treated with alemtuzumab
	• Treated with teriflunomide in 12 months prior to screening
	• Prior exposure to fingolimod or natalizumab within 90 days, or glatiramer acetate, interferons, dimethyl fumarate, or glucocorticoids within 30 days
	• Pregnant or nursing
	• ≥ 10 years disease duration with subjects EDSS ≤2.0
	• Contraindication for MRI and gadolinium
	• Known presence of neurologic disorders that may mimic MS
	• Current or known history of clinically significant infection
	• History of clinically significant CNS trauma
	• History of medically significant adverse effects from corticosteroids, diphenhydramine, or antibodies
	• Absolute neutrophil or platelet count outside of normal range
	• Absolute lymphocyte counts < 1000/μl
	• Any severe and/or uncontrolled medical conditions or other conditions that could affect their participation in the study
	• Current participation in any other interventional clinical trial
	• Inability or unwillingness to comply with study and/or follow-up procedures outlined in the protocol
	• Lack immunity to varicella as determined by screening
	• Vaccination with live virus in past 2 months

antigens. Thus, B cells are likely far more important in presenting self-antigens to autoreactive T cells than macrophages and dendritic cells. This concept is supported by the observation that B cell-specific MHC-II-deficient mice are resistant to the development of experimental autoimmune encephalomyelitis (EAE; Molnarfi et al., 2013), a CNS autoimmune disease in mice reminiscent of MS. B cells also produce cytokines that influence the microenvironment and function of other immune cells. MS patients have been shown to have B cells with enhanced expression of pro-inflammatory cytokines that may contribute to pathology (Bar-Or et al., 2010). Another possibility is that B cells are harboring a virus, such as EBV, that may be contributing to MS pathology and thus, elimination of B cells may be eliminating a key regulator of pathogenesis.

Ublituximab is a third generation, glycoengineered chimeric anti-CD20 antibody being developed for B cell malignancies and MS (Sawas et al., 2017). It is designed to have enhanced affinity for CD16, FcγRIIIa receptors, facilitating more efficient NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) of CD20+ B cells (Le Garff-Tavernier et al., 2014). In the present study, peripheral blood mononuclear cells (PBMCs) were collected from relapsing MS patients enrolled in a phase IIa safety and dose-finding study of ublituximab (TG1101-RMS201), and immune profiles were analyzed over a 24 week period. The goal of this study was to determine if monitoring lymphocyte subsets following B cell depletion with ublituximab could shed light on the possible mechanism of action of B cell depletion in MS. Importantly, the patients in this study had a significant clinical improvement with reduction in lesion burden and relapse rate (Fox et al., 2018).

2. Materials and methods

2.1. Patients

Forty-eight relapsing MS patients were enrolled in a phase IIa clinical trial (TG1101-RMS201) to test the efficacy of ublituximab to deplete B cells, as well as determine the optimal infusion protocol. Patients were consented and enrolled at Hope Neurology MS Center, Knoxville, TN ($n = 14$; Chesapeake IRB); The Ohio State University Wexner Medical Center, Columbus, OH ($n = 10$; Western IRB); SC3 Research Group, Pasadena, CA ($n = 9$; Chesapeake IRB); Associates in Neurology, Lexington, KY ($n = 5$; Chesapeake IRB); Central Texas Neurology Consultants, Round Rock, TX ($n = 4$; Chesapeake IRB); University of Colorado Anshutz Medical Campus, Aurora, CO ($n = 5$; Western IRB); and Neurology Center of San Antonio, San Antonio, TX ($n = 1$; Chesapeake IRB). Inclusion and exclusion criteria are listed in Table I. There were 32 female and 16 male patients enrolled with a mean age of 39.2 years. Mean disease duration was 7.4 years with 22 patients (46%) diagnosed within 5 years, 10 patients (21%) diagnosed in 5–10 years, and 16 patients (33%) diagnosed > 10 years. PBMCs were collected at 11 time points: screening (week 0), baseline (week 1), week 1 day 2 (24 h after treatment with ublituximab), and weeks 2, 3, 4, 8, 12, 16, 20 and 24. All patients received 150 mg ublituximab immediately following the baseline blood collection, and either 450 mg ($n = 24$) or 600 mg ($n = 24$) ublituximab at week 3 (14 days after initial dose). One patient was omitted from the PBMC analysis due to a modified treatment schedule, so 47 patients were included in the immune profile analysis. No patients discontinued treatment during the 24 weeks. This study was approved by either Western IRB or Chesapeake IRB for each site as noted above, and informed consent was provided by every patient.

2.2. Flow cytometry

Blood samples were collected in heparinized tubes, insulated, and shipped overnight to Columbus, Ohio. Peripheral blood mononuclear cells (PBMC) were isolated over a Ficoll gradient, washed in PBS, and resuspended in medium [RPMI 1640 (Corning), 5% HI-human serum (Sigma-Aldrich), 1% (HEPES, L- glutamine, Pen/Strep)]. Four flow cytometry antibody panels were used to analyze the lymphocyte populations. Panel A included: V450-CD3 (UCHT1), PECy7-CD19 (SJ25C1), and FITC-CD56 (NCAM16.2). Panel B included: V450-CD3 (UCHT1), V500-CD4 (L200), PECy7-CD8 (RPA-T8), APC-CD45RA (HI100), and APCH7-CD27 (M-T271). Panel C included: V450-CD3 (UCHT1), V500-CD8 (RPA-T8), APCH7-CD27 (M-T271), APC-CD45RA (HI100), FITC-IL10 (BT-10), PE-IFNγ (4S.B3), PerCPCy5.5-GMCSF (BVD2-21C11), and PECy7-IL17 (BL168). Panel D included: V450-CD3 (UCHT1), V500-CD4 (L200), FITC-CD25 (M-A251), and PE-FOXP3 (PCH101). For panel C, the PBMCs were stimulated with 50 ng/ml of PMA (Sigma cat#16561–29-8) and 1 μg/ml of ionomycin (Sigma cat#I0634), and treated with 0.2ul of Golgi plug (BD cat#555029) for 4 h prior to

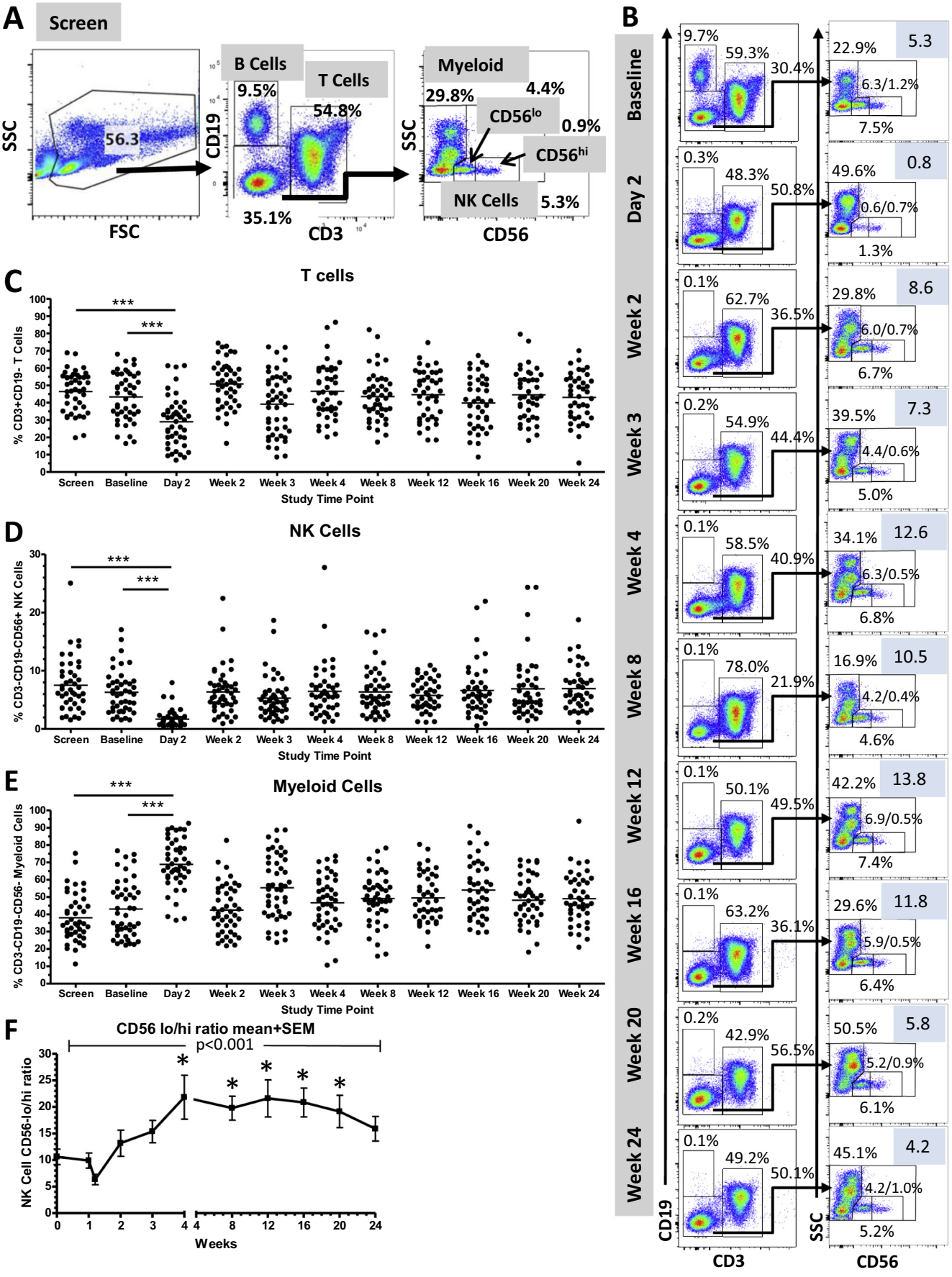

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Fig. 1. Ublituximab efficiently depletes B cells with minimal effects on the homeostatic levels of T cells, NK cells and myeloid cells. Blood was collected from 47 relapsing MS patients prior to (week 0/screen and week 1/baseline) and following (week 1 day 1 and weeks 2, 3, 4, 8, 12, 16, 20 and 24) ublitxumab treatment. Flow cytometry was used to identify and monitor the percentage of B cells, T cells, NK cells and myeloid cells. (A) The gating strategy to identify the cell populations is shown for the screen (week 0) for a representative patient, including identification of highly cytolytic ($CD56^{lo}$) and less cytolytic ($CD56^{hi}$) NK cells. (B) The baseline through week 24 flow cytometry data for the same patient in A is shown. The blue box shows the $CD56^{lo}$ to $CD56^{hi}$ ratio. The percentage of T cells (C), NK cells (D), and myeloid cells (E) for all 47 patients is shown. The significant change in each population from screen and baseline is shown (***$p < .001$). (F) The percentage of $CD56^{lo}$ and $CD56^{hi}$ NK cells was determined for each time point for each patient and the ratio of $CD56^{lo}$ to $CD56^{hi}$ NK cells was determined for each time point for each patient. The mean ± SEM of the ratios is shown. Mixed Model for Repeated Measures was used to determine if there was a significant change during the entire 24 week period which is shown above the graph. *$p < .05$ time point compared to screen and baseline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

staining. For staining, 1×10^6 cells were washed and resuspended in cold PBS [1% BSA], and incubated with FcR Blocking Reagent (Miltenyi) at 4 °C for 10 min. Antibodies were then added to a working volume of 100 µl in PBS [1% BSA], and incubated for 30 min at 4 °C. Samples were washed, and fixed at 4 °C with either: PFA [0.5%], Cytofix/Cytoperm Solution Kit (BD Biosciences), or FOXP3 Transcription Factor Staining Set (eBioscience). Fixed cells were stained for intracellular molecules in their appropriate permeabilization wash buffers, and incubated for 30 min at 4 °C (cytokines) or 45 min at room temperature (Foxp3). Finally, cells were washed and resuspended in PBS [1% BSA]. Cytometric data was acquired and analyzed using BD FACSCanto II, FACSDiva, FlowJo, and Microsoft Excel.

2.3. Statistical analysis

Mixed Model for Repeated Measures was used to analyze changes in the cell population data over the 24 weeks (IBM SPSS v.25). In addition, a modified One Way ANOVA for Repeated Measures was employed in which missing data values ($n = 0$–4 per time point) were estimated using the variance in the means of each time point and normalizing to the preceding value for that specific individual. This model was validated by comparing the p values of the modified ANOVA and Mixed Model for Repeated Measures which were consistent. Bonferroni's Multiple Comparison Test was then used to determine which specific time points were significantly different than screen/baseline (GraphPad Prism 4).

3. Results

3.1. Ublituximab efficiently depletes B cells from the circulation

Flow cytometric analysis was utilized to identify the B cell (CD19+), T cell (CD3+), and NK cell (CD56+) populations over time (Fig. 1A-B). The analysis was performed on two pre-treatment samples, one during the initial screening of the patient for enrollment in the trial and again on the day of their first infusion (baseline), and 9 post-treatment time points. The mean time between screen (week 0) and baseline (week 1) was 22.1 days. The gating strategy is shown in a representative flow cytometric analysis which defined the B, T, and NK cell populations, as well as the $CD56^{lo}$ and $CD56^{hi}$ NK cell subsets (Fig. 1A-B). While there was a defined B cell population at screening (Fig. 1A) and baseline (Fig. 1B top), on day 2 (~24 h after the infusion of ublituximab) the number of B cells was significantly reduced (Fig. 1B). Patients received a second dose of ublituximab at week 3. No significant recovery of B cells during the 24 week monitoring period was seen, illustrating that ublituximab rapidly and efficiently depletes peripheral blood B cells in MS patients (Fox et al., 2018).

3.2. Ublituximab transiently alters the T cell:NK cell: myeloid cell ratio

Depletion of a specific cell type, such as B cells, in peripheral blood would be expected to result in a relative increase in the other cell types. However, analysis of CD3+ T cells and CD3-CD19-CD56+ NK cells revealed that the percentage of T cells and NK cells in the PBMCs was significantly reduced at day 2, ~ 24 h after ublituximab infusion, but normalized by week 2 (Fig. 1B, C, D). In contrast, the percentage of myeloid cells significantly increased at day 2 (Fig. 1E), suggesting that there may be an efflux of myeloid cells from the bone marrow in response to the rapid depletion of B cells. Thus, the reduction in the percentage of T cells and NK cells may be due to a relative increase in the number of myeloid cells in the peripheral blood and not an actual reduction in the number of these cells. While a very small percentage of T cells express CD20 (Holley et al., 2014; Palanichamy et al., 2014; Schuh et al., 2016), the significant reduction in the percentage of T cells at baseline (43.3% ± 13.8) compared to day 2 (29.0% ± 14.3) cannot be explained by ublituximab-mediated depletion of CD20+ T cells.

NK cells are not known to express CD20 so direct depletion of NK cells is unlikely. However, the glycosylation modifications made to ublituximab make it highly susceptible to NK-mediated ADCC (Hauser et al., 2008). To further define the changes in the NK cell population during ublituximab therapy, the $CD56^{lo}$ and $CD56^{hi}$ NK cells were analyzed (Fig. 1A, B, F). The blue box in Fig. 1B shows the $CD56^{lo}$:$CD56^{hi}$ ratio for this representative patient. Analysis of the ratio of $CD56^{lo}$ to $CD56^{hi}$ NK cells at day 2 found that there was a disproportional loss of $CD56^{lo}$ cells, the NK cells that express high levels of CD16 (FcγRIII), compared to $CD56^{hi}$ cells (Fig. 1B, F). Since ublituximab is designed to elicit NK-mediated ADCC via CD16 engagement, this suggests that the cytolytic NK cells ($CD56^{lo}$) were partially depleted following ublituximab therapy, consistent with activation-induced NK cell apoptosis following the NK cells lysing of anti-CD20-bound B cells. While the percentage of total NK cells normalized by week 2 (a week following ublituximab treatment; Fig. 1D), the ratio of $CD56^{lo}$ to $CD56^{hi}$ NK cells continued to rise and was significantly elevated week 4 through week 20, indicating that there was a skewing of the NK cell population in response to ublituximab treatment (Fig. 1F). The $CD56^{lo}$:$CD56^{hi}$ NK cell ratio returned to normal range by week 24.

3.3. B cell depletion increases the ratio of naïve to memory T cells

To determine if B cell depletion altered the T cell populations over time, we analyzed the total CD4+ and CD8+ T cell populations, as well as naïve and memory T cell subsets. The percentage of total CD4 and CD8 T cells changed significantly during the 24 week period (Fig. 2 and 3A). The percentage of total CD4 and total CD8 T cells declined immediately following ublituximab therapy (Fig. 2C and 3A), consistent with the total T cell data (Fig. 1C). The total CD4 T cells normalized by week 3 and the total CD8 T cells normalized by week 2, and both remained in normal range through week 24.

To determine if there were changes in naïve and memory CD4+ and CD8+ T cell subsets, CD45RA (a naïve T cell marker) and CD27 (a costimulatory molecule expressed by naïve and central memory T cells) expression were analyzed on CD4+ (Fig. 2) and CD8+ (Fig. 3) T cells (Appay et al., 2008; Tonaco et al., 2017). The gating strategy is illustrated in Fig. 2A-B for CD4+ T cell subsets, and the same strategy was used for CD8+ T cell subsets (not shown). The percentage of naïve CD4+ T cells (CD3 + CD4 + CD8-CD45RA + CD27+; Fig. 2D) significantly increased, and both the CD4+ effector memory (CD3 + CD4 + CD8-CD45RA-CD27-; Fig. 2F) and central memory T cells (CD3 + CD4 + CD8-CD45RA-CD27+; Fig. 2G) declined following B cell depletion. Although the CD4+ effector T cells

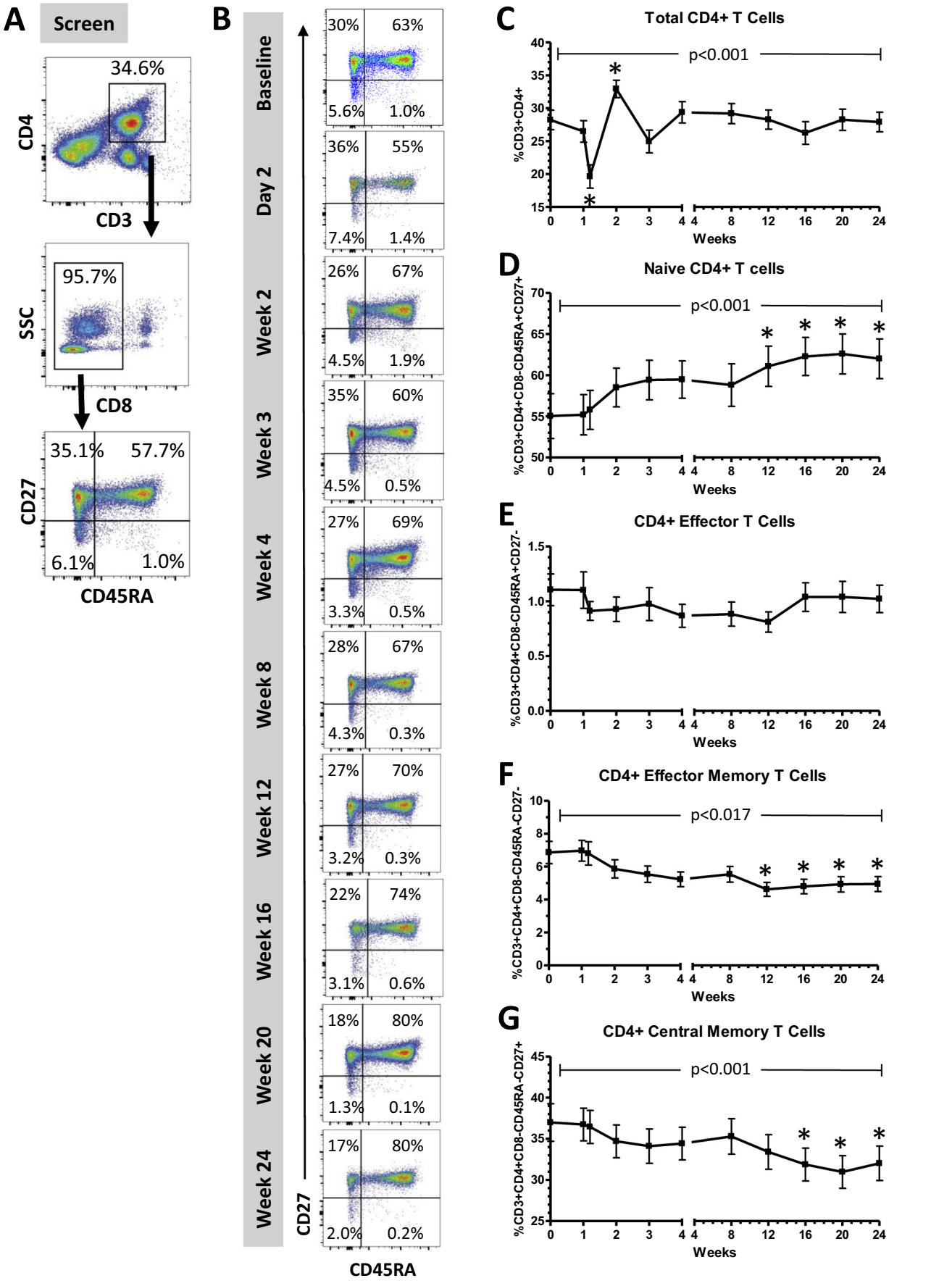

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Fig. 2. Ublituximab treatment shifts the balance of naïve and memory CD4 + T cells. (A) The gating strategy for identifying the CD4 + T cell subsets is shown for the screen (week 0) for a representative patient. The naïve CD4 + T cells were defined as CD3 + CD4 + CD8-CD45RA + CD27 + (upper right quadrant), new effector CD4 + T cells were CD3 + CD4 + CD8-CD45RA + CD27- (lower right quadrant), CD4 + effector memory T cells were CD3 + CD4 + CD8-CD45RA-CD27- (lower left quadrant), and CD4 + central memory T cells were CD3 + CD4 + CD8-CD45RA-CD27 + (upper left quadrant). (B) Flow cytometry analysis of the CD4 + T cell subsets from the same representative patient in A for Baseline (week 0) through week 24. The mean ± SEM for total CD4 + T cells (C), naïve CD4 + T cells (D), CD4 + new effector T cells (E), CD4 + effector memory T cells (F), and CD4 + central memory T cells is shown for all 47 patients. Mixed Model for Repeated Measures was used to determine if there was a significant change during the entire 24 week period which is shown on the top of each graph. *$p < .05$ time point compared to screen and baseline.

(CD3 + CD4 + CD8-CD45RA + CD27-) appeared to decline initially and return to normal levels by week 16, no significant change was seen in this population at any time point (Fig. 2E). The fact that no significant change in any CD4 + T cell subset (Fig. 2D-G) at day 2 suggests that the apparent decline in the percentage of total CD4 + T cells at day 2 is likely an artifact due to the increase in myeloid cells (Fig. 1E).

The net effect of B cell depletion on CD8 + T cells was very similar to CD4 + T cells with a gradual, yet significant, increase in the percentage of naïve CD8 + T cells (CD3 + CD8 + CD4-CD45RA + CD27 +; Fig. 3D), and reciprocal decrease in CD8 + effector memory (CD3 + CD8 + CD4-CD45RA-CD27-; Fig. 3F) and central memory T cells (CD3 + CD8 + CD4-CD45RA-CD27 +; Fig. 3G). One notable difference between the CD4 + and CD8 + T cell analysis was the change in the ratio of naïve to memory T cells immediately following ublituximab treatment on day 2. While there was an apparent loss of effector and memory CD8 + T cell populations, an increase in naïve CD8 + T cells was seen at day 2. The fact that this occurred in the CD8 + T cells, but not the CD4 + T cells, suggests that some memory CD8 + T cells may have been deleted by ublituximab, which is consistent with prior literature demonstrating that CD20 + T cells are primarily memory CD8 + T cells (Schuh et al., 2016).

3.4. B cell depletion diminishes Th1 cells while enhancing Tregs

The CD4 + T cells were further analyzed to determine if the phenotype of the CD4 + T cell had been changed following B cell depletion with ublituximab. The PBMCs for 7 time points were stimulated with PMA/ionomycin for 4 h to promote the expression of cytokines already programmed in the memory CD4 + T cells. The percentage of IFNγ +, IL-17 +, GM-CSF + and IL-10 + cells in the CD3 + CD4 + CD45RA- memory T cells was determined (Fig. 4). A significant decline in the percentage of Th1 (IFNγ +; $p < .001$; Fig. 4A-B), but not in Th17 (IL-17 +; $p = .117$; Fig. 4C), and GM-CSF + CD4 + T cells ($p = .057$; Fig. 4D) was observed, although the mean percentage of these CD4 + T cell subsets did decline over the 24 week time period. Very few CD4 + IL-10 + T cells were seen and no change in the frequency of IL-10 + CD4 + T cells was observed (data not shown).

Given that it has previously been reported that there is a defect in Tregs in MS patients (Viglietta et al., 2004; Haas et al., 2005; Huan et al., 2005; Kumar et al., 2006; Haas et al., 2007; Venken et al., 2008), we analyzed this population as well. The percentage of CD4 + $CD25^{hi}$Foxp3 + T cells (Tregs) in the peripheral blood is typically very low; however, the percentage of Tregs significantly increased over time in MS patients treated with ublituximab (Fig. 4E). These data indicate that the potentially pathogenic T cells were diminishing, while the beneficial Tregs were increasing following B cell depletion.

4. Discussion

The goal of this study was to monitor the lymphocyte profile in patients on ublituximab therapy to further our understanding about mechanism of action of B cell depletion therapy in MS. Ublituximab efficiently depleted peripheral B cells in the patients in this study and the patients had an improved clinical outcome (Fox et al., 2018). Within 24 h of the first dose of ublituximab, the percentage of B cells declined from a mean of 7.3% to 0.2%, and this level of B cell depletion was maintained for the 24 week study period. Concordant with the rapid depletion of B cells at 24 h was a significant decrease in the percentage of T cells and NK cells immediately following ublituximab administration, accompanied by an increase in the percentage of myeloid cells (Fig. 1C-E). One would expect if the B cells were depleted, a relative increase would be seen in all other peripheral blood cell populations, since flow cytometry is based on the percentages of a specific cell population within the myeloid/lymphocyte gate. The decrease in NK cells is not surprising. Ublituximab has been engineered to increase the binding affinity of the Fc portion of the antibody to CD16, the FcγRIIIa receptor on NK cells, and enhance NK cell-mediated ADCC of the ublituximab-bound B cells. Following NK cell-mediated lysis of the B cells, NK cells likely die via activation-induced apoptosis (Jewett and Bonavida, 1996). $CD56^{lo}$ NK cells which express high levels of CD16 were depleted to a greater extent than the $CD56^{hi}$ NK cells which have low expression of CD16 (Cooper et al., 2001). $CD56^{lo}$ NK cells, which are lytic, likely became exhausted and succumbed to activation-induced apoptosis. The $CD56^{lo}$ NK cells recovered within a week and were expanded relative to the $CD56^{hi}$ NK cell population, skewing the ratio of $CD56^{lo}$ to $CD56^{hi}$ NK cells for several weeks.

It is less clear why there was an increase in myeloid cells and a decrease in T cells. Myeloid cells may be increased due to an efflux of myeloid cells from the bone marrow to fill the void created by the rapid loss of B cells. An increase in myeloid cells would decrease the percentage of other cell types, such as T cells, without necessarily affecting the absolute number of T cells in the blood. However, a small subset of T cells expresses CD20 and thus, may be susceptible to depletion by ublituximab. CD20 + T cells have been seen in the blood and brain of MS patients and express proinflammatory cytokines (Holley et al., 2014; Palanichamy et al., 2014; Schuh et al., 2016), suggesting that CD20 + T cells have pathogenic potential. The frequency of CD20 + T cells is higher in MS patients, they are predominantly CD8 memory T cells, and anti-CD20 therapy effectively depletes these T cells (Palanichamy et al., 2014; Schuh et al., 2016). Our study showed that the percentage of total T cells was 45% pre-ublituximab and dropped to 29% within 24 h after treatment, a far more significant decrease than would be expected by depletion of CD20 + T cells. The fact that there was no selective depletion of naïve or memory CD4 + T cell subsets is more indicative of a relative change in the percentage of total CD4 + T cells in the peripheral blood and not an actual depletion of CD4 + T cells. In contrast, while the total percentage of CD8 + T cells decreased at week 1 day 1 (Fig. 3A), similar to the total CD4 + T cells, the percentage of naïve CD8 + T cells increased (Fig. 3B), and the effector and memory CD8 + T cells decreased (Fig. 3C-E). These findings suggest that preferential loss of a small CD20 + effector/memory CD8 + T subset may have occurred, which would be consistent with the observation that CD20 + T cells are highest in the memory CD8 + T cell subset (Schuh et al., 2016). Importantly, the T cells, NK cells, and myeloid cells returned to normal levels within a week, while the B cells remained depleted for the 24 weeks. The return of T cells, NK cells, and myeloid cells to homeostatic levels suggests that patients would respond appropriately to most immunological challenges, which is consistent with previous studies indicating that infection rates and severity are not significantly changed in MS patients on B cell depleting therapy (Hauser et al., 2008; Bar-Or et al., 2008; Hawker et al., 2009; Kappos et al., 2011; Montalban et al., 2017; Hauser et al., 2017).

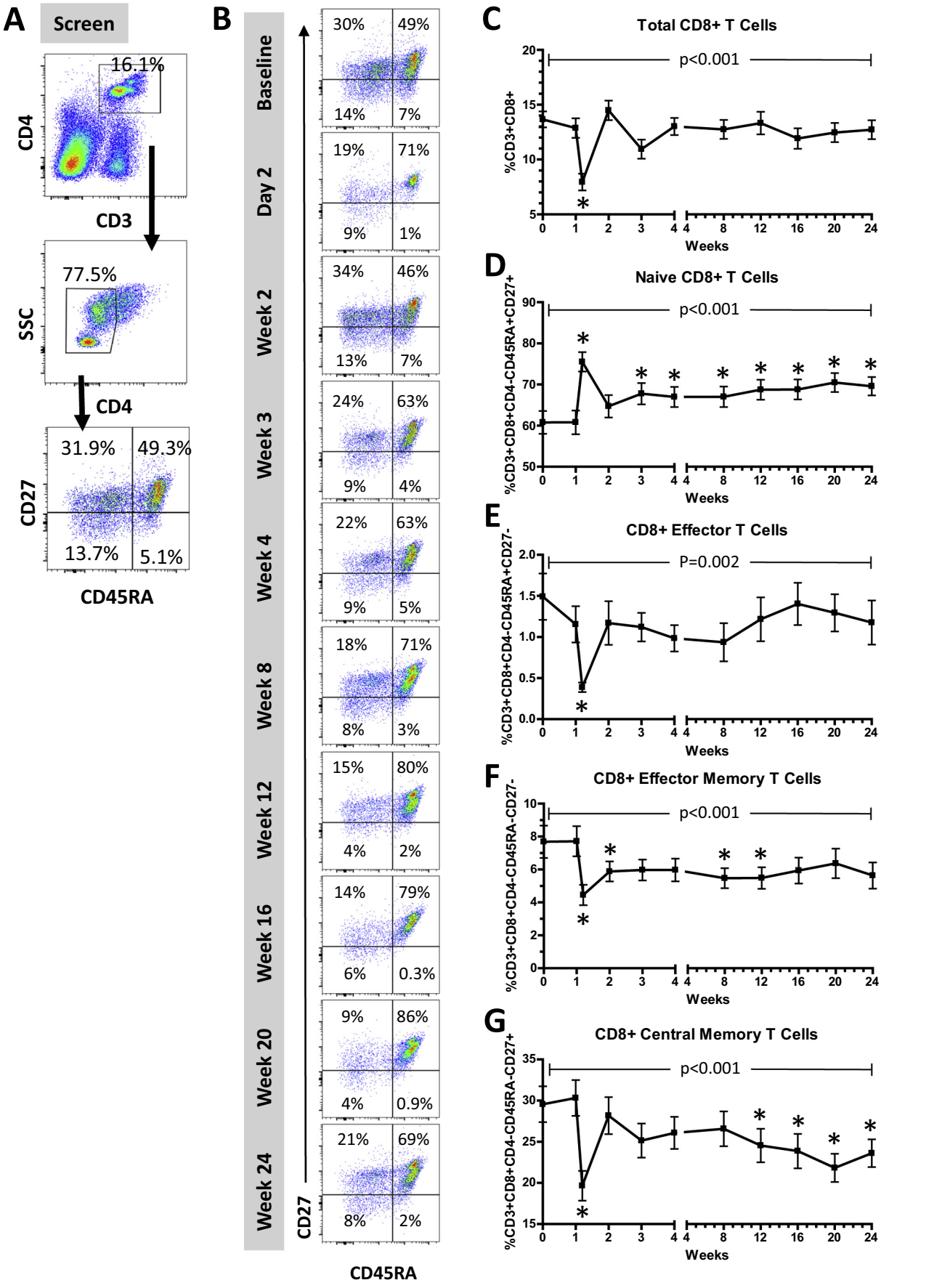

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Fig. 3. Ublituximab treatment shifts the balance of naïve and memory CD8+ T cells. (A) The gating strategy for identifying the CD8+ T cell subsets is shown for the screen (week 0) for a representative patient. The naïve CD8+ T cells were defined as CD3 + CD8 + CD4-CD45RA + CD27+ (upper right quadrant), new effector CD8+ T cells were CD3 + CD8 + CD4-CD45RA + CD27- (lower right quadrant), CD8+ effector memory T cells were CD3 + CD8 + CD4-CD45RA-CD27- (lower left quadrant), and CD8+ central memory T cells were CD3 + CD8 + CD4-CD45RA-CD27+ (upper left quadrant). (B) Flow cytometry analysis of the CD8+ T cell subsets from the same representative patient in A for Baseline (week 0) through week 24. The mean ± SEM for total CD8+ T cells (C), naïve CD8+ T cells (D), CD8+ new effector T cells (E), CD8+ effector memory T cells (F), and CD8+ central memory T cells (G) is shown for all 47 patients. Mixed Model for Repeated Measures was used to determine if there was a significant change during the entire 24 week period which is shown on the top of each graph. *$p < .05$ time point compared to screen and baseline.

One of the most striking observations in this study is the change in CD4+ T cells. While the percentage of new effector CD4+ T cells is not significantly different over the course of the 24 weeks, the number of naïve CD4+ T cells transitioning to effector T cells and then to memory T cells is reduced. Previous studies have shown that both healthy controls and MS patients have similar numbers of myelin-specific T cells in the blood, but myelin-specific T cells in MS patients have a memory phenotype (Allegretta et al., 1990; Lovett-Racke et al., 1998). Thus, improving the naïve to memory CD4+ T cell ratio would likely reduce the number of pathogenic T cells in MS patients. While dendritic cells are very efficient APCs in the activation of naïve CD4+ T cells, dendritic cells have no specificity for antigen and require recognition of antigen as foreign. In contrast, B cells present antigen that has specificity for their B cell receptor, and thus have the capacity to present specific antigens to a T cell with that same antigen specificity. B cells require relatively little antigen to activate CD4+ T cells and are thought to be particularly important in the activation of autoreactive T cells, because the B cell receptor cannot differentiate between self and foreign antigens. Dendritic cells and macrophages must be initially activated by engagement of pattern recognition receptors via pathogens to become efficient APCs. Thus, dendritic cells and macrophages would be poor presenters of self-antigens in the absence of pathogens. In EAE, it has been shown that CNS B cells are critical for the reactivation of T cells in the CNS, and the failure of CNS B cells to activate myelin-specific T cells mitigates recruitment of immune cells into the CNS (Pierson et al., 2014). The decrease in CD4+ memory T cells following ublituximab treatment would suggest that the loss of B cells is reducing the activation of CD4+ T cells and supports the hypothesis that B cell depletion eliminates a critical APC population.

Similar to CD4+ T cells, a gradual increase in naïve CD8+ T cells and a decrease in memory CD8+ T cells is following ublituximab treatment. CD4+ T cells play a major role in the activation and expansion of CD8+ T cells by up-regulating costimulatory molecules and producing IL-2. The reduction in CD4+ T cell activation would result in less activation of CD8+ T cells, and an increase in the naïve to memory CD8+ T cell ratio. CD8+ T cells are abundant in MS lesions, but the role of these cells in the pathogenesis of MS is still unclear. However, they have the capacity to lyse MHC class I+ cells, such as oligodendrocytes, and thus reducing the CD8+ memory T cells has the potential to reduce CNS pathology in MS patients. A reduction in both CSF and peripheral blood T cells had been observed in MS patients treated with rituximab indicating that T cells are being modulated by B cell depletion (Cross et al., 2006; Piccio et al., 2010.

Myelin-specific CD4+ T cells are thought to be the primary orchestrator of pathology in MS. Th1, Th17 and CD4 + GM-CSF+ T cells have all been implicated in mediating CNS autoimmunity (Lovett-Racke et al., 2011; Baecher-Allan et al., 2018). Ublituximab treatment resulted in a significant decrease in Th1 cells, and a trend in decreasing in Th17 cells and CD4 + GM-CSF+ T cells, indicating that the potentially pathogenic CD4+ T cell population was being reduced. The observation that Th1 cells, but not Th17 cells, were significantly reduced may be due to the fact that Th1 cells are more abundant and thus easier to detect changes in this population. Alternatively, Th1 cells may be more dependent on B cells as APCs, but EAE studies found that B cell-specific deletion of MHCII resulted in a significant reduction in both Th1 and Th17 cells, suggesting that B cells contribute significantly to both of these Teff populations (Molnarfi et al., 2013). Importantly, the CD4+ Treg population expanded and was significantly increased by week 20. By week 20–24, 31/47 (66%) of MS patients had an increase > 25% in their CD4+ Treg population and 20/47 (42%) of patients had more than doubled the percentage of CD4+ Treg cells. Most previous studies that analyzed Tregs in MS found a defect in Treg function, and a less prominent defect in Treg number (Viglietta et al., 2004; Haas et al., 2005; Huan et al., 2005; Kumar et al., 2006; Haas et al., 2007; Venken et al., 2008). Thus, determining if Treg suppressive function is restored in MS patients following B cell depletion will be important in future studies. It is also important to note that most of the significant changes in the T cell subsets occurred after 12 weeks and some as late as 20 weeks, consistent with a gradual shift in the T cell profile from effector/memory to more naïve as would be expected if B cells are critical to the activation of autoreactive effector/memory T cells. Fluctuations in the T cell subsets over time in the MS patients are evident, indicating that the patients are still responding to antigens (Fig. 3B). It will be interesting to determine if there is a differential T cell response to myelin compared to pathogens in patients on B cell depletion to determine if B cells are more critical for presentation of self antigens compared to environmental antigens.

The potential benefit of B cell depletion extends beyond antigen presentation. First, while B cells produce both pro- and anti-inflammatory cytokines, MS patients' memory B cells express higher amounts of TNF, LTα, IL-6 and GM-CSF, all known to enhance CNS inflammation (Li et al., 2018). Following B cell depletion with rituximab in MS, the B cells that returned were largely naïve and pro-inflammatory cytokine production was reduced (Li et al., 2018). Second, there is evidence that B cells from MS patients express soluble, non-cytokine, proteins that are toxic to oligodendrocytes and neurons (Lisak et al., 2012; Lisak et al., 2017). Third, B cells harbor viruses, such as EBV, that has been associated with the development of MS and MS progression (Laurence and Benito-Leon, 2017; Burnard et al., 2017), so depletion of B cells would potentially reduce the primary reservoir for EBV. Finally, there has been significant interest in meningeal B cell follicles that develop in some MS patients with secondary progressive MS (Serafini et al., 2004; Magliozzi et al., 2007; Howell et al., 2011). Cytokines produced by B cells are critical for the development of B cell follicles, so it is likely that B cell depletion would minimize the formation of meningeal B cell follicles is likely. However, the change in T cell subsets following B cell depletion observed in this study suggests that B cells are important APCs in MS, and depletion of B cells reduces T cell activation and shifts the balance of naïve, effector and memory T cells.

To our knowledge this study is the first comprehensive, longitudinal analysis of the lymphocyte profile in MS patients following B cell depletion therapy. Overall, the data illustrates a favorable shift in the T cell profile, reducing the memory pro-inflammatory T cell subsets, and expanding the naïve and Treg populations. The rapid restoration of T cell:NK cell:myeloid cell ratio following ublituximab therapy would suggest that immune homeostasis is largely intact in the absence of B cells. Plasma cells and antibody titers are largely unaffected by B cell depletion therapy, and several studies have demonstrated that patients treated with B cell depleting antibodies retain memory B cells and the ability to generate an antibody response to vaccines, although it may not be as robust (Kim et al., 2013; Cho et al., 2017; Stokmaier et al.,

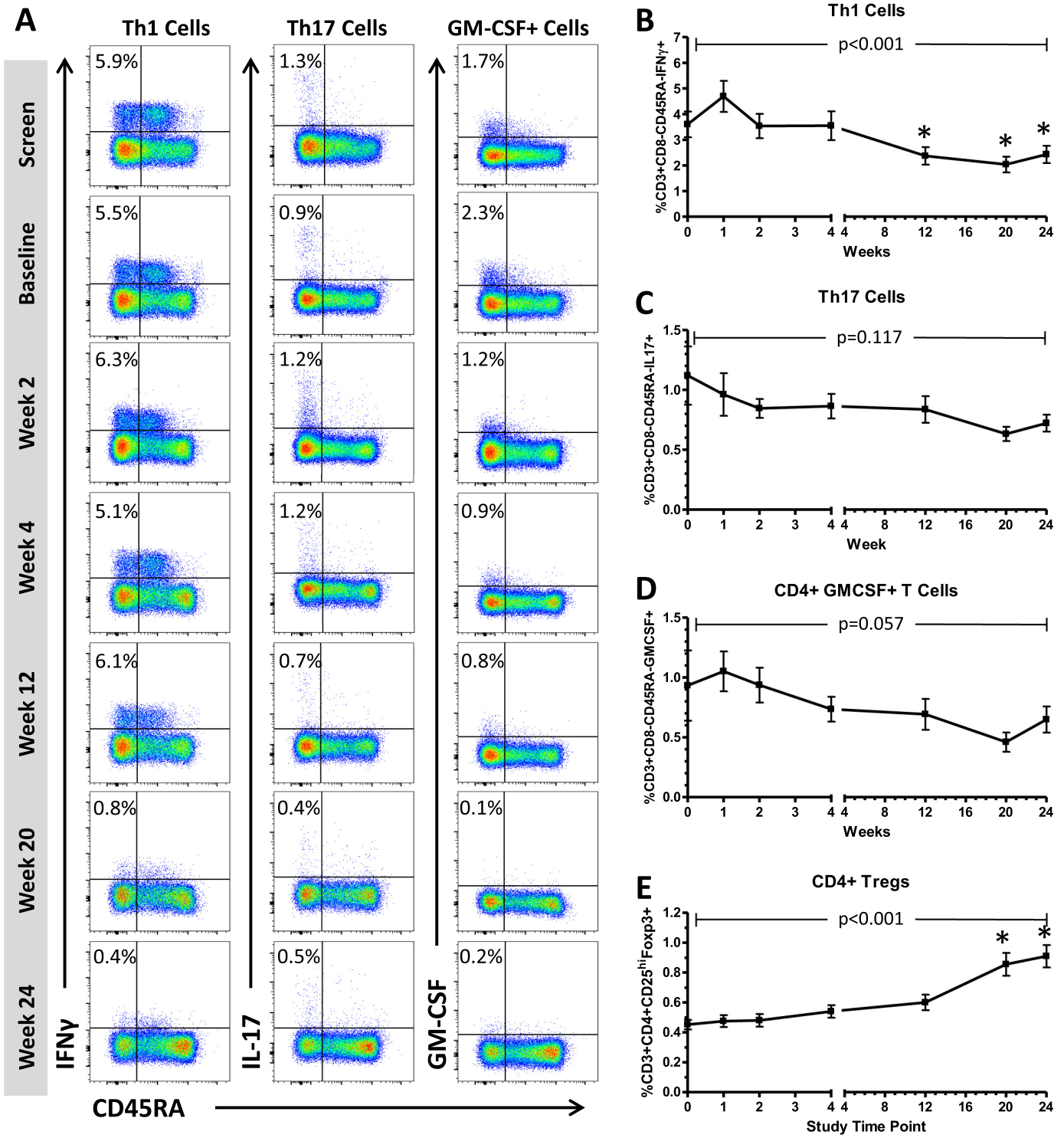

Fig. 4. Ublituximab reduces the percentage of Th1 cells and increases the percentage of Tregs over time. (A-D) PBMCs from each patient at 7 time points were stimulated with PMA/ionomycin for 4 h in vitro and then analyzed for intracellular production of IFNγ, IL-17, and GM-CSF. Th1 cells were defined as CD3 + CD8-CD45RA-IFNγ+, Th17 cells were defined as CD3 + CD8-CD45RA-IL17+, and CD4+ GM-CSF+ T cells were defined as CD3 + CD8-CD45RA-GMCSF+. (A) Flow cytometry data from a representative patient. The upper left quadrant shows this population from screen through week 24 for Th1 cells (left column), Th17 cells (middle column), and CD4+ GM-CSF+ T cells (right column). The mean ± SEM for the 47 patients is shown for Th1 cells (B), Th17 cells (C), and CD4+ GM-CSF+ T cells (D). (E) CD4+ Tregs were also analyzed at these same time points and were defined as CD3 + CD4 + $CD25^{hi}$Foxp3+. The mean ± SEM is shown for all 47 patients. Mixed Model for Repeated Measures was used to determine if there was a significant change during the entire 24 week period which is shown on the top of each graph (B-E). *$p < .05$ time point compared to screen and baseline.

2018). This indicates that there are still naïve and memory B cells in the lymphoid tissues that are capable of differentiating into antibody-producing cells and responding to infections. In future studies, analyzing absolute cell numbers of the T cell subsets will be important; however, the effectiveness of the immune system is dependent on balancing the effector and regulatory components and thus understanding changes in the relative percentages of various lymphocyte subsets is valuable. Analyzing the normal fluctuation in T cell subsets in healthy individuals would be beneficial in determining if MS patients on B cell depletion therapy restore Teff/Treg ratios to normal levels. It will also be important to study changes in self-reactive T cells, to fully understand how B cell depletion is changing the T cell landscape over time.

Potential conflicts of interest

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