Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter know, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Jakob G. Habib April 17, 2013

Peripheral Regulatory B Cell Phenotype in Multiple Sclerosis Patients

By

Jakob G. Habib

Jacques Galipeau, MD

Adviser

Department of Biology

Jacques Galipeau, MD

Adviser

Christopher Beck, PhD

Committee Member

Barry Yedvobnick, PhD

Committee Member

José Soria, PhD

Committee Member

April 17, 2013

Peripheral Regulatory B Cell Phenotype in Multiple Sclerosis Patients

By

Jakob G. Habib

Jacques Galipeau, MD

Adviser

An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2013

Abstract

Peripheral Regulatory B Cell Phenotype in Multiple Sclerosis Patients
By Jakob G. Habib

Multiple Sclerosis (MS) is a demyelinating autoimmune disease. Though the exact pathogenesis of the disease is not clear, both T cells and B cells play key roles. B regulatory cells (Bregs) are a small subset of B cells that produce the anti-inflammatory cytokine interleukin 10 (IL-10). We found that there is an increase in B cell number and Naïve B cell number in MS patients (1347 ± 159 cells/μL, average ± SEM) compared to healthy controls (935±129 cells/μL). Our lab has created a fusion protein of granulocyte macrophage colony stimulating factor and interleukin 15, named GIFT15, which induces a Breg phenotype in naïve B cells. Treatment of peripheral blood mononuclear cells with GIFT15 results in proliferation of B cells and increased production of IL-10. These findings establish a baseline of B cell phenotype in MS patients and essential for future therapeutic treatments of MS with GIFT15.

Peripheral Regulatory B Cell Phenotype in Multiple Sclerosis Patients

By

Jakob G. Habib

Jacques Galipeau, MD

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2013

Acknowledgements

This material is funded by a National Institute of Health R01 grant (1R01AI093881-01A1) to Jacques Galipeau, MD. I am forever indebted to Dr. Galipeau, my Principal Investigator, for his generous support, wisdom and advice.

I'd also like to thank Dr. Christopher Beck, my undergraduate biology adviser and professor, who opened the doors to me in biological research.

Further, great thanks extend to Dr. Neil Lava for his contribution of Multiple Sclerosis blood samples and his instruction in MS pathology. Additional thanks to Dr. William Tyor and Dr. Edmund Waller's lab.

Special thanks to everyone in Dr. Galipeau's lab for their strong support and encouragement. A particular thanks to Dr. Jiusheng Deng for his laboratory instruction and tutelage over the past two years. Thanks to Ms. Shala Yuan and Dr. Andrea Pennati for their assistance with GIFT15.

Finally, I would like to thank my mother and brothers for their love, support, and encouragement.

Table of Contents

List of Figures

List of Tables

Introduction

Overview of Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune disease caused by inflammatory damage to the myelin and axons in the Central Nervous System (CNS). Myelin functions as an electrical insulator that covers nerve axons and serves to increase the speed of action potential conduction.[1] The disease is more prevalent in women and frequently manifests between the ages of 20-40. Both environmental and genetic factors are important in disease onset and progression, but the underlying pathogenesis is unknown.[2]

Vitamin D is both a modulator of calcium homeostasis and an immune enhancer. Studies indicate that Vitamin D supplements are therapeutic to individuals with MS and mice with Experimental Autoimmune Encephalomyelitis (EAE), the murine model of MS.[3] Cigarette smoking increases the risk of MS, and smoking during pregnancy increases the risk of a child developing MS.[3] Epstein-Barr Virus (EBV) infection is also associated with MS. EBV, a herpes virus, is often nonsymptomatic but lies dormant in memory B cells over one's lifetime and can trigger continuous stimulation of the immune system; the risk of developing MS is 10 times lower in individuals negative for EBV.[3]

Linkage analysis and association studies have historically been used to determine genes involved in MS pathogenesis.[4] These techniques have revealed disease activity through the human leukocyte antigen (HLA) class II locus within the MHC (major histocompatibility complex) region on chromosome 6.[5] HLA molecules are found on antigen presenting cells (APCs), and present bound peptides for T cell activation.[6] Autoimmune damage to myelin occurs when cluster of differentiation 4 ($CD4^+$) T cells interact with epitopes of myelin proteins through HLA class II molecules.[7] While HLA

haplotypes have been associated with MS, a specific gene locus has yet to be connected with the disease.[4]

The symptoms of MS can vary widely between patients based on where demyelinating lesions occur. Doctors use neurological exams and magnetic resonance imaging (MRIs) to diagnose patients and to track disease progression over time. Multiple MRIs over several months may be required to show multiple lesions at different locations in different points of time. The presence of oligoclonal bands or increased levels of immunoglobulin G (IgG) in cerebral spinal fluid (CSF) can also facilitate diagnosis when MRIs are inconclusive.[2]

MS can be divided into different types of disease: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), and secondary progressive MS (SPMS). RRMS is the most common form, encompassing 80% of patients. Symptoms typically develop over several days, stabilize, and then usually improve within weeks. PPMS involves a gradual, steady progression in disease activity and includes 20% of patients. In addition, 70% of RRMS patients eventually develop SPMS, which, like PPMS, is characterized by steady disease progression between relapses.[8]

Currently the only drug treatments for MS affect RRMS disease progression. The drug Natalizumab (Tysabri) decreases the frequency of relapses and slows disability progression by preventing the migration of lymphocytes from the periphery into the CNS.[9] Interferons (Avonex and Betaseron) mediate the immune system by altering cytokine production, inhibiting T-cell activation, and decreasing the expression of MHC Class II molecules.[10] Glatiramer acetate (Copaxone) mediates the disease progress of RRMS

patients by shifting Th1 cells to Th2 cells (T helper cells). These Th2 cells secrete anti-inflammatory cytokines such as interleukin 5 (IL-5) and interleukin 13 (IL-13).[11]

B Cell Subsets

Until recently, MS has traditionally been described as a T cell mediated disease. However, studies have shown that B cells have a strong effect on the activity of MS. B cells are 2.5**%** percent of lymphocytes and 10% of peripheral blood mononuclear cells (PBMCs), though these frequencies vary between individuals.[12] B cells function by producing immunoglobulin, functioning as APCs, and secrete pro- and anti-inflammatory cytokines.[13] Depletion of peripheral B cells with the drug rituximab leads to a rapid decline of disease activity in MS.[14,15] B cells mature in the bone marrow, can circulate through the blood and lymphatic system, and are identifiable by their expression of CD19. Naïve B cells, identifiable by their expression of Immunoglobulin D (IgD), can differentiate into memory B cells when activated by either T cell dependent or independent antigens. These memory B cells are long-lived, specific to the initially encountered antigen, and identifiable by their expression of CD27.[16] Normally, B cell development in the bone marrow prevents the formation of B cells that react to self-antigens. Abnormal development occasionally results in the formation and release of auto-reactive B cells, which plays a role in autoimmune diseases.[17]

While the majority of B cells stimulate the immune system, some B cells suppress immune function. These B regulatory cells (Bregs) are a small subset of B cells with $CD1d^{hi}CD5^{+}$ surface markers.[18] They produce a diversity of immunomodulating mediators, with the most predominant and well-established factor being interleukin 10 (IL-10). IL-10

broadly suppresses the immune system by modulating the function of innate and adaptive responses. It prevents monocytes and macrophages from producing pro-inflammatory cytokines, such as interleukin 1 (IL-1) and Tumor Necrosis Factor (TNF), and it also inhibits $CD4^+$ T cell proliferation and cytokine production.[19]

GIFT15

In Dr. Galipeau's lab we develop non-synthetic fusion cytokines (fusokines) by combining two different naturally occurring cytokines, often resulting in novel immune functions. We developed a granulocyte macrophage colony-stimulating factor (GMCSF) and interleukin 15 (IL-15) fusion transgene (GIFT15) with novel immunosuppressive properties. (Figure 1). GMCSF and IL-15 are both immune-stimulatory cytokines. GMCSF initiates the production of IL-1 and TNF-α and is crucial for the development of APCs.[20] IL-15 activates and stimulates the proliferation of T cells and NK cells. [21,22] The IL-15 receptor has three chains: α, β, and γ. IL-15 binds to the α chain and activates Janus kinase (JAK) which phosphorylates Signal Transducer and Activator of Transcription (STAT). IL-15 normally stimulates both the β chain (JAK1/STAT3) and the γ chain (JAK3/STAT5). However, when GIFT15 binds to the α chain, the GMCSF component blocks γ chain signaling and results in hyperphosphorylation of STAT3 in relation to STAT5.[20] STAT3 has survival, proliferative, angiogenic, and immunosuppressive functions.[20] In addition, this asymmetrical STAT3/STAT5 phosphorylation ultimately induces the differentiation of naïve B cells into induced Bregs (iBregs).[23,24] A diagram of this signaling pathway is shown in Figure 1.

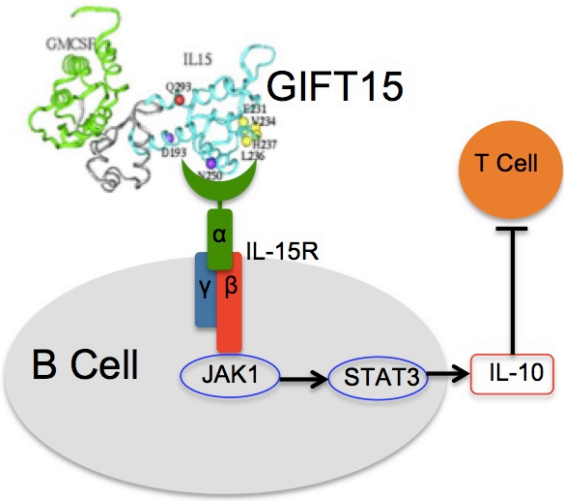

Figure 1. Signaling pathway of GIFT15.

The function of iBregs has previously been demonstrated in EAE: administration of iBregs robustly inhibits EAE progression and induces complete recovery from EAE.[20] Splenic B cells obtained from mice are treated with GIFT15 and the resulting iBregs are injected back into mice, attenuating EAE.[24] Following these murine studies, we will treat purified B cells from MS patients with GIFT15 and examine the production of anti-inflammatory cytokines in iBregs. Our final goal is to harness this quality of GIFT15 to generate large amounts of autologous iBregs for personalized immunotherapy to treat MS patients. Before human trials can commence, a B cell baseline in MS patients must first be established. In this study we determine the phenotype and frequency of B cells in MS patients and examine the effect that GIFT15 has on PBMCs from MS patients. We hypothesize that the total B cell number, and IL-10 producing Breg subset in particular, of both peripheral blood B cell populations and GIFT15 treated PBMCs is distinct when comparing MS to healthy controls.

Aims

The following are experimental goals followed by the methods used to accomplish them:

1. Establish a phenotypic baseline of B cells, specifically regulatory B cells, to determine whether B cell populations are distinct between MS and Healthy Controls (HC).
    a) Flow cytometric analyses of cell surface markers (CD19, CD1d, CD5, CD27, IgD) to define B cell subsets in whole peripheral blood.
2. Culture PBMCs with GIFT15 and quantify the amount of IL-10 produced.
    a) Spectroscopic quantification of IL-10 protein by enzyme-linked immuno sorbent assay (ELISA).
3. Identification of expanded IL-10 producing iBregs.
    a) Measure proliferative capacity with carboxyfluorescein succinimidyl ester (CFSE).
    b) Flow cytometric analysis of cell surface markers and intracellular IL-10 to functionally characterize expanded iBregs.

Methods

Sample Collection

31 MS blood samples were collected from consenting adults at the Emory MS clinic by our collaborators: Dr. Neil Lava and Dr. William Tyor. The sex, age, disease state, and MS drugs taken were recorded for each donor. 34 healthy controls (HC) blood samples were

provided by Dr. Edmund Waller's lab. The institutional review board (IRB) protocol has been approved by Emory University.

Reagents

All antibodies were purchased from Biolegend (CD19-PE/Cy7, CD5-FITC, CD1d-PE, CD27-APC, IL-10-PE, and IgD-PerCP/Cy5.5). Recombinant human GMCSF and IL-15 were purchased from R&D Systems. Capture and detection antibodies against human IL-10 and GMCSF were purchased from Thermo Scientific.

GIFT15 Production

293T cells transfected to produce human GIFT15 were cultured at 37°C, 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% p/s in 250 cm^2 culture dishes for 2 days. The media was then changed to FBS free DMEM (with 1% p/s) and incubated for 1 day at 37°C, 5% CO_2. Cells were collected and column concentrated with Centricon Plus-70 Filter Units (Millipore). GMCSF ELISA was utilized to determine the GIFT15 concentration.

PBMC Isolation

PBMCs were isolated by ficoll density gradient separation. Blood was diluted 1:2 with Dulbecco's Phosphate-Buffered Saline (DPBS) and gently layered on top of 5mL Ficoll (Corning) in a 15mL tube. The sample was centrifuged at 1500RPM at 20°C for 20 minutes with the break off. The PBMC layer was carefully isolated and washed in 50mL DPBS. PBMCs were then suspended in 10mL complete RPMI (RPMI-1640 containing 10% fetal

bovine serum (FBS), 1% penicillin/streptomycin (p/s), 1% MEM nonessential amino acids, 1% HEPES, 0.1% β-mercaptoethanol). PBMCs were stained with trypan blue and enumerated using a hemocytometer. PBMCs were then cultured and excess cells were cryopreserved.

PBMC Culture

200µL PBMCs at a concentration of $1x10^6$ cells/mL were placed in a 96 well tissue culture plate. To each well, 293T conditioned media (CM), GIFT15 (10ng/mL), or a mix of recombinant GMCSF and IL-15 (10ng/mL) was added and the cells were incubated at 37°C, 5% CO_2 for 4 days. The supernatant was collected and stored at -20°C for ELISA.

B Cell Subset Analysis

Whole blood samples (3-5mL) were collected in EDTA (Vacutainer company). 100µL whole blood samples were stained with 5µL anti-CD19, anti-CD1d, anti-CD5, and anti-CD27, and 2µL anti-IgD for 20 minutes at room temperature (RT) in the dark. Following staining, 500µL of red blood cell (RBC) lysis buffer containing tris buffered ammonium chloride was added and the tube was lightly vortexed every 2 minutes for 10 minutes. 500µL of Phosphate-Buffered Saline (PBS) was then added, followed by 50µL AccuCheck Counting Beads (Invitrogen) to quantify the B cell subsets. The sample in its entirety was processed by flow cytometry on a BD FACS Canto II machine. Data was collected on BD FACS Diva software and analyzed with FlowJo. Distinct B cell subsets were defined utilizing the gating strategy indicated in Figure 2.

ELISA

ELISAs for IL-10 and GMCSF followed the protocol for Thermo Scientific. Capture antibodies, detection antibodies, and avidin-HRP were diluted 1:250. The plate was coated with capture antibody overnight and washed with TBST 5 times between each step.

CFSE

Carboxyfluorescein succinimidyl ester (CFSE) staining followed the protocol from BD. HC PBMCs were stained with CFSE at a concentration of 5μM. The CFSE labeled PBMCs were incubated with CM, GIFT15, or a mix of GMCSF and IL-15 for 4 days as aforementioned. As cells divide the concentration of CFSE decreases with each generation.

Intracellular Cytokine Staining

Intracellular cytokine staining (ICS) was performed on HC PBMCs. In order to measure the stimulatory effects of GIFT15, Phorbol-Myristate-Acetate (PMA) and Ionomysin were not added. PBMC culture was performed and incubated for 72 hours as aforementioned. Brefeldin A (BFA) was then added (10μg/mL) and incubated at 37°C, 5% CO_2 for 12 hours. The ICS protocol for Molecular Probes was then followed. Cells were stained extracellularly for CD19 and intracellularly for IL-10.

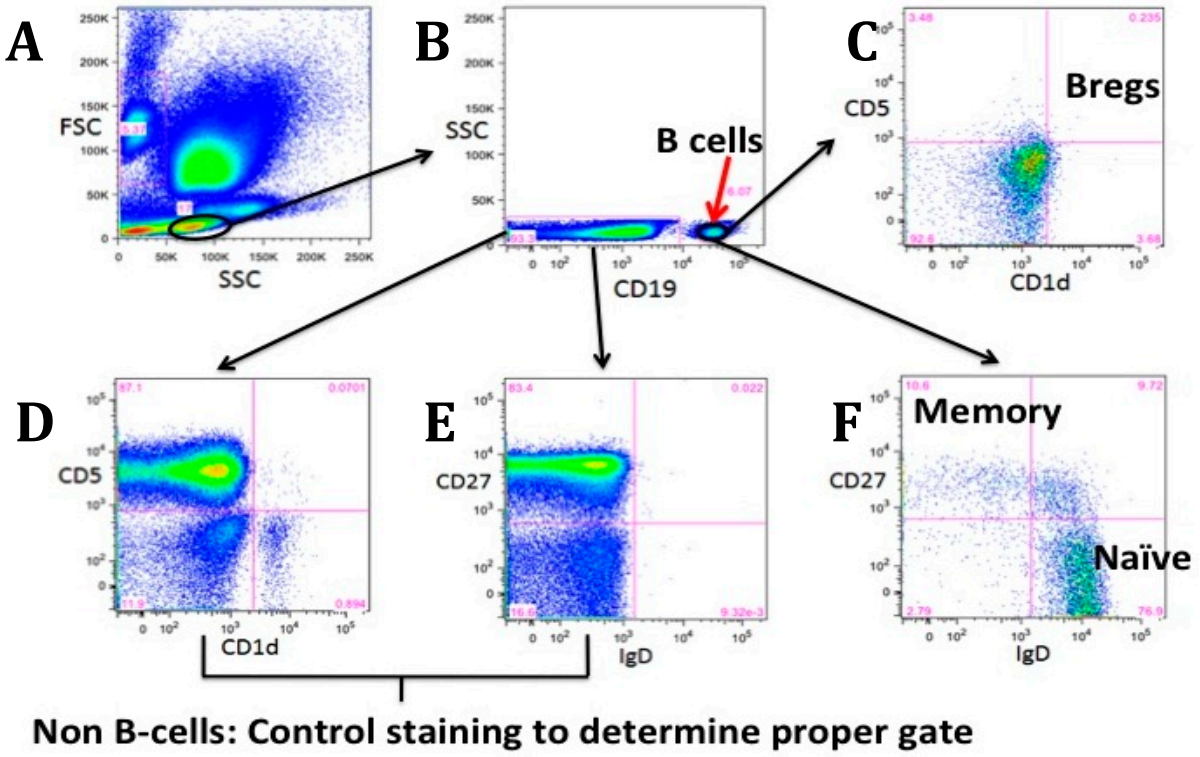

Figure 2. Whole blood gating strategy. **(A)** Forward scatter (FSC) measures cell size and side scatter (SSC) measures cell granularity. **(B)** Lymphocyte gating revealed $CD19^{+}$ (B cells) and $CD19^{-}$ populations. **(D)** $CD19^{-}$ population analysis revealed distinct CD5 and CD1d and **(E)** CD27 and IgD populations. These population delineations were used to determine gating on B cells for **(C)** CD5 and CD1d and **(F)** CD27 and IgD.

Statistical Analysis

Student's two-tailed t-test assuming unequal variance was used to determine significance with $p<0.05$. Values were expressed as average ± standard error of the mean (SEM). Error bars display the SEM. The formula below was used to calculate the absolute number (N) of total B cells or B cell populations per µL whole blood:

$$\text{Absolute Count}\left(\frac{\text{cells}}{\mu\text{L}}\right) = \frac{\text{\# of cells counted}}{\text{Total \# of beads counted}} \text{ X } \text{\# of Counting Beads}\left(\frac{\text{beads}}{\mu\text{L}}\right)$$

Results

Whole Blood Analysis

We found a significant increase in the number of B cells in MS, 1347±159 cells/μL compared to healthy controls (HC), 935±129 cells/μL (p=0.049, Figure 3A). We did not find a difference in the number of Bregs, memory B cells, or naïve B cells between MS and HC (Figure 3B, C, D). However, we did find a significant increase (p=0.035) in the number of naïve Bregs in MS, 4.7±0.9 cells/μL compared to HC, 2.3±0.7 cells/μL (Figure 3E). In order to determine whether this increase in the number of naïve Bregs was a result of the increased number of total B cells, we also compared the percent of naïve B cells that were $CD1D^{hi}CD5^{+}$. We found a significant increase (p=0.004) in the percent of naïve Bregs between MS, 0.59±0.08% and HC, 0.32±0.05% (Figure 3F).

Patient Subset Analysis

Figure 4 shows the number of MS patients on each drug. Of the 31 patients, 29 had RRMS, one patient had PPMS and one patient had SPMS. Table 1 lists the frequency of each B cell population for no medication, Glatiramer Acetate, and Interferon. No significant difference was found within these subsets or between these subsets and HC.

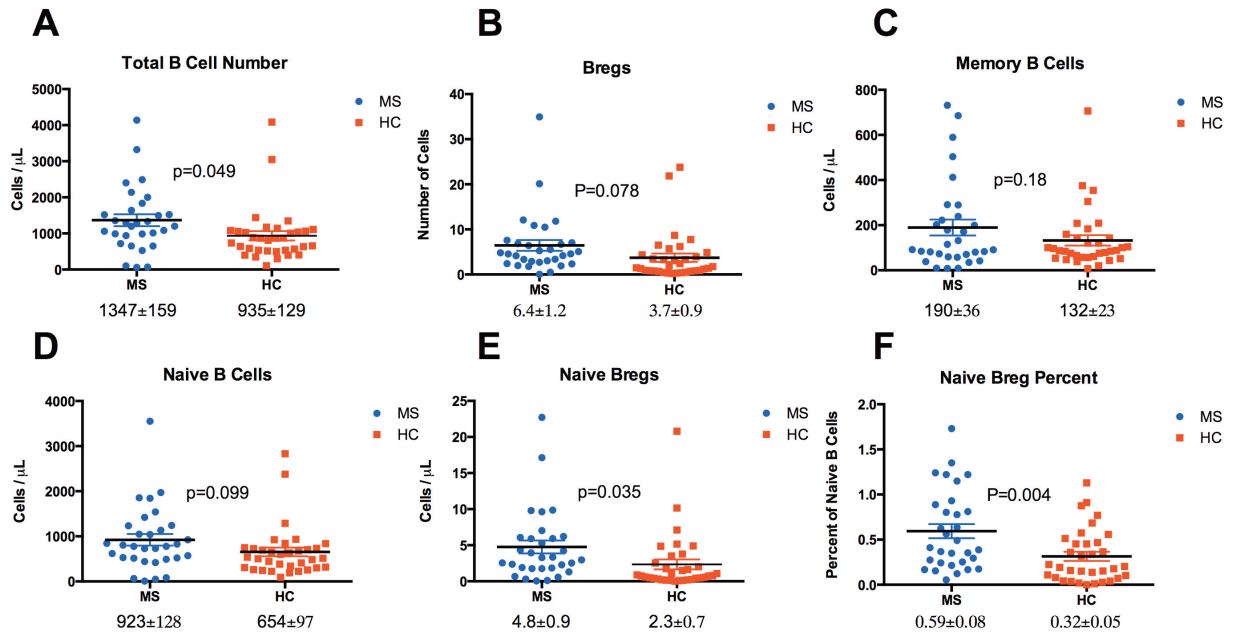

Figure 3. Analysis of whole blood samples for MS and HC. **(A)** MS patients have significantly increased B cell number, p=0.049. **(B-D)** Breg, memory B cell, and naïve B cell distributions show no significant difference. **(E-F)** MS patients have more naïve Bregs, p=0.035, and a significantly increased percentage of $CD1d^{hi}CD5^{+}$ naïve B cells, p=0.004.

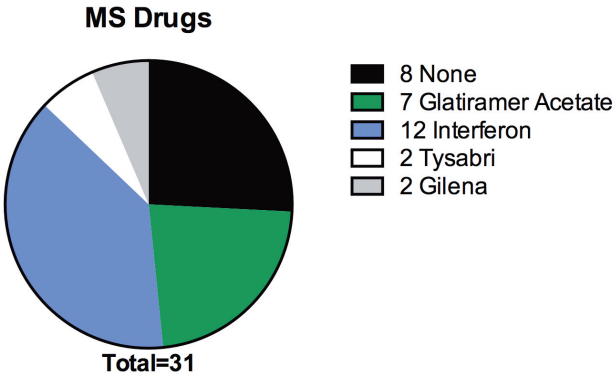

Figure 4. MS patient drug distributions.

Table 1. Subset analysis of MS patients on no medication, Glatiramer Acetate, and Interferon. Values are expressed in cells/μL. No significant difference was observed within these subsets and between each subset and HC. ($p>0.05$ for each value.)

Subset	B cells	Bregs	Memory B Cells	Naïve B Cells	Naïve Bregs
No Medication	1439±399	5.0±0.7	170±66	1067±362	4.1±0.7
Glatiramer Acetate	1167±186	5.7±1.2	195±56	786±109	4.4±1.0
Interferon	1426±207	6.6±1.6	170±57	1003±183	4.9±1.5

PBMC Culture

The IL-10 concentration in PBMC supernatant treated with CM, GIFT15, or a mix of GMCSF and IL-15 was measured by ELISA. We determined experimentally that incubation for 3-4 days resulted in optimal IL-10 concentration (Figure 5). An increase in IL-10 concentration was observed for GIFT15 treated PBMCs compared to both CM and GMCSF + IL-15 in both MS (n=2) and HC (n=2) (Figure 6). The IL-10 concentration for CM and GMCSF + IL-15 was below the detection limit (7 pg/mL) for one of the HC groups.

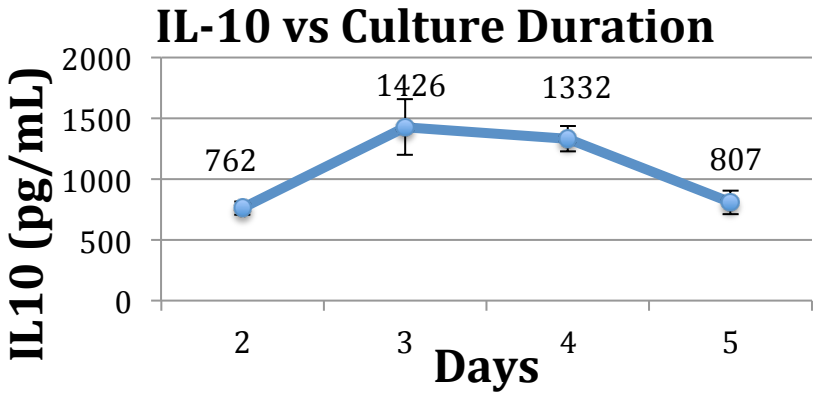

Figure 5. IL-10 concentration in GIFT15 PBMC supernatant peaks at 3 days.

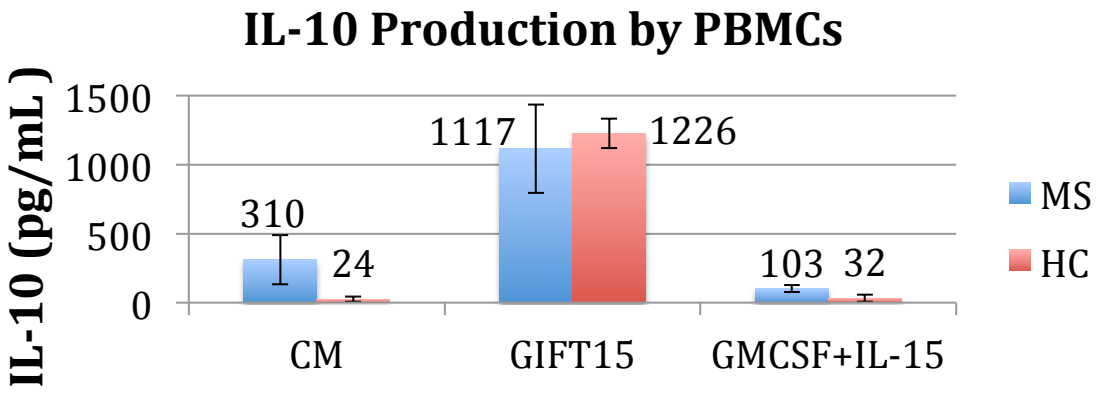

Figure 6. The concentration of IL-10 significantly increases in the supernatant of GIFT15 cultured PBMCs. n=2 for MS and HC.

CFSE Proliferation

In order to verify that GIFT15 induced proliferation, we measured the proliferation of HC PBMCs with CFSE. We found that GIFT15 induced the proliferation of $CD19^+$ B cells (Figure 7). GIFT15 did not induce proliferation in $CD19^-$ lymphocytes. However, the mixture of GMCSF and IL-15 did induce minor proliferation in $CD19^-$ lymphocytes (Figure 8).

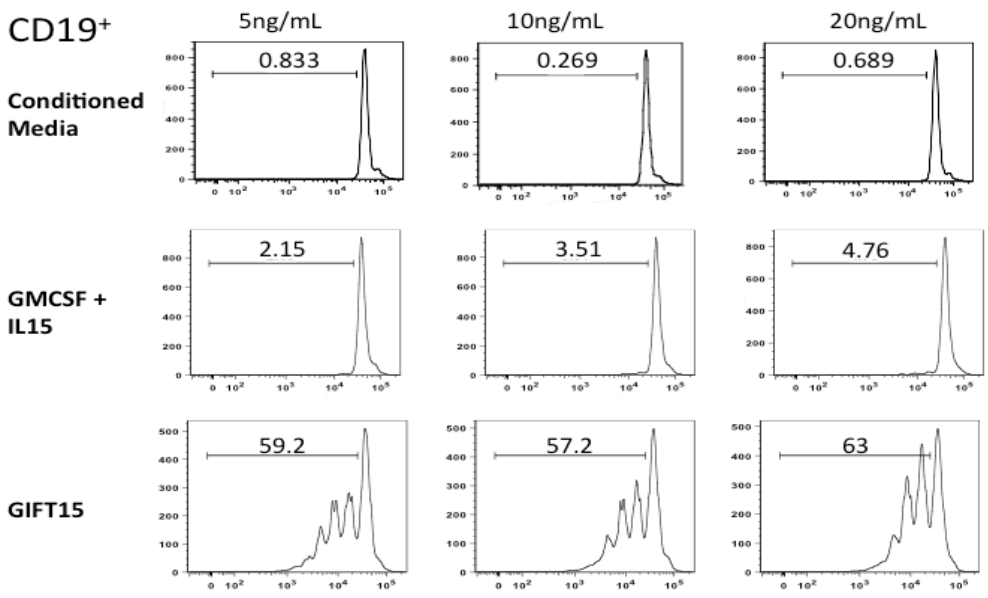

Figure 7. CFSE proliferation assay of HC PBMCs shows expansion of GIFT15 cultured $CD19^+$ lymphocytes. Each subsequent peak represents a replication cycle. x-axis: CFSE; y-axis: number of cells

Intracellular Cytokine Staining

We sought to characterize the IL-10 content of GIFT15 treated PBMCs and as a preliminary experiment measured the intracellular content of HC PBMCs stimulated with CM, GIFT15, and GMCSF + IL-15. We detected a shift of increased IL-10 cells in the $CD19^{+}$ GIFT15 cultured PBMCs that is not observed in CM or GMCSF + IL-15 cultured cells (Figure 9A). We did not observe this shift in $CD19^{-}$ lymphocytes (Figure 9B).

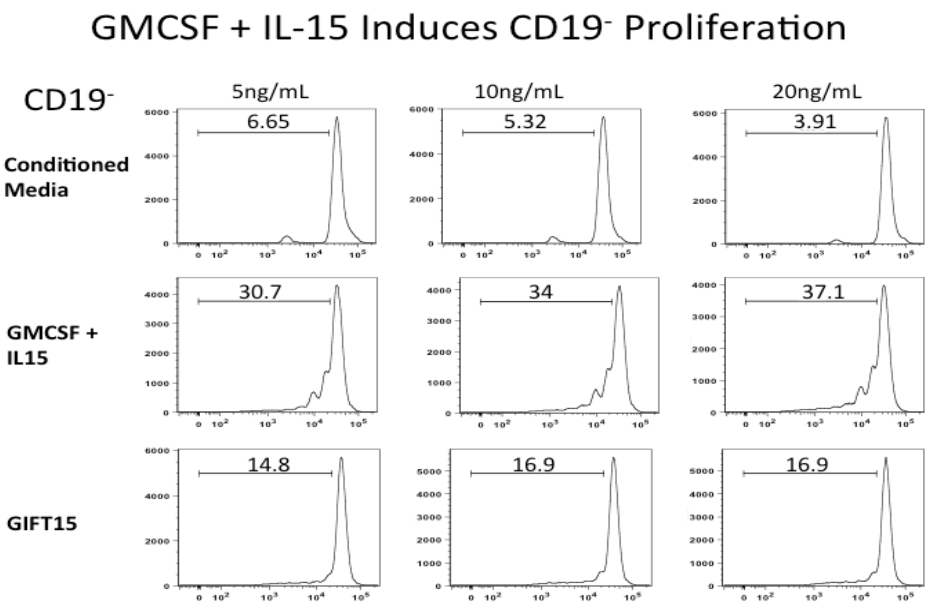

Figure 8. CFSE proliferation assay of HC PBMCs shows a slight expansion of GMCSF + IL-15 cultured $CD19^{-}$ lymphocytes. x-axis: CFSE; y-axis: number of cells

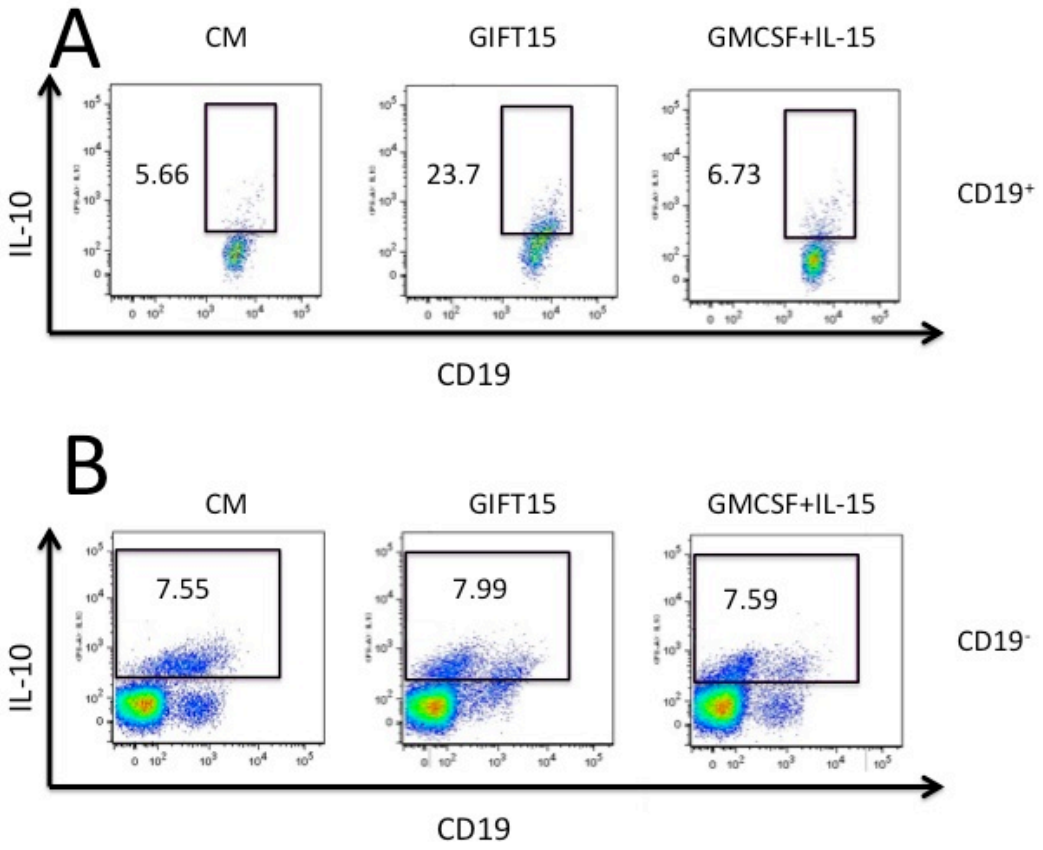

Figure 9. ICS of HC PBMCs cultured in CM, GIFT15, or GMCSF + IL-15. (**A**) $CD19^+$ lymphocytes reveal a shift of increased IL-10 content in GIFT15 cultured cells (**B**) not seen in $CD19^-$ cells. x-axis: CD19; y-axis: IL-10

Discussion

Accomplishment of Aims

The majority of MS research over the years has focused primarily on T cells. However, recent studies have revealed the importance of B cells in MS pathogenesis.[14] Because the precise role of B cells in MS is not yet fully understood, we investigated the phenotype of peripheral B cells in MS patients. We first characterized B cells by examining different populations of B cells in peripheral whole blood using flow cytometry. Our findings demonstrate that MS is not associated with a significant deficiency in Bregs (Figure 3B). We found an increase in total B cell number (Figure 3A) and naïve Breg number (Figure 3E) in MS patients. The percent of naïve B cells with a Breg phenotype increased in MS as well (Figure 3F), demonstrating that this increase in naïve Breg number is not directly related to the increase in total MS B cells.

These observations, along with previous studies, lead us to suggest that there is a correlation between altered proportions of naïve and regulatory B cell subsets and MS pathogenesis.[25,26] It is possible that the increased number of B cells, coupled with no significant increase in Breg number, is related to MS pathogenesis. B cells are primarily involved in MS disease progression; it is the small subset of Bregs that have immunosuppressive capabilities. Memory B cells can cross the blood-brain barrier where they undergo maturation and differentiation, and populations of B cells have been found in MS lesions.[14,25] In addition, B cell depletion therapies have proven effective in treating MS.[14] The small number of endogenous Bregs might not sufficiently counter the effect of the elevated number of B cells in MS patients, which is why administration of autologous GIFT15 iBregs might be a therapeutic treatment for MS.

We did not find a significant difference between patients not taking drugs, those taking Glatiramer Acetate, and those taking Interferons (Table 1). Patients taking Gilena and Tysabri were excluded because only two samples from each were available. The Interferon category (Figure 4) is a grouping of different types of interferons including Avonex and Betaseron. As we continue this study and obtain a larger sample size, especially for patients on Gilena and Tysabri, further subset analysis will be performed and any notable differences will be taken into account.

The results of our second aim show that PBMCs cultured with GIFT15 have increased IL-10 production (Figure 6). The results in Figure 6 utilize only two samples for both MS and HC. As that sample size is expanded it is possible that we will observe a difference in IL-10 production between GIFT15 treated MS and HC PBMCs. Studies have shown that B cells in MS patients produce less IL-10, and that IL-10 is produced mainly by naïve B cells.[14,25] Therefore, the increased IL-10 concentration for MS CM was an interesting and unexpected finding. With the sample size currently so small it might represent an outlier, but it might instead reflect increased basal IL-10 production in MS PBMCs. The sample with an increased CM IL-10 concentration was from a patient not taking immunomodulating drugs, while the other sample was from a patient taking Gilena and had a CM IL-10 concentration similar to that measured in HC. As the sample size of cultured PBMCs increases we will determine whether this difference in CM IL-10 concentration is maintained.

To accomplish the third aim of this study we further examined the effects of GIFT15 on PBMCs. Using CFSE we determined that GIFT15 induces proliferation of B cells (Figure 7) but not $CD19^-$ lymphocytes (Figure 8). This $CD19^-$ lymphocyte population contains T

cells, and literature showing that IL-15 induces proliferation of certain T cell populations might explain the slight proliferation in GMCSF + IL-15 treated PBMCs (Figure 8).[27] After confirming that GIFT15 induces B cell proliferation in PBMCs, we investigated whether these B cells also produced increased IL-10. This quantification of IL-10 by flow cytometry requires ICS because IL-10 does not present as a surface cytokine. Our pilot experiment showed that there is indeed a shift in IL-10 producing B cells (Figure 9A) that is not seen in $CD19^-$ lymphocytes (Figure 9B). However, the isotype stain for Figure 9 did not successively delineate between positive and negative IL-10 populations (data not shown) so the experiment must therefore be repeated with the isotype fluorescent antibody issue resolved.

Future Direction

Before *in vivo* GIFT15 trials can commence, the effect of GIFT15 iBregs on other factors in the immune system must be established. Trials with the B cell depletion drug Rituximab have shown to reduce the number of lesions and relapses in RRMS patients.[9] Though the exact mechanism of disease attenuation by Rituximab is not fully understood, it clearly shows that B cells play a key role in MS. It is possible that a future treatment of MS might involve both GIFT15 and rituximab. B cells isolated from PBMCs of an MS patient could be treated with GIFT15 to induce a Breg phenotype. These autologous iBregs could then be administered back to the patient after Rituximab therapy. In this way, GIFT15 and Rituximab synergistically ameliorate MS by drastically increasing the number of IL-10 producing Bregs in the patient. In addition, future studies could explore the possibility of utilizing GIFT15 as a therapy for different B cell mediated inflammatory diseases.

Conclusion

In this study we determined the phenotype and frequency of B cells in MS patients, finding that patients with MS have an increased number of B cells and naïve Bregs. We also measured the effects of GIFT15 on PBMCs, observing increased proliferation and IL-10 production. Our findings from this study are important for establishing a phenotypic baseline for B cells in MS patients. Our ultimate goal is to utilize the inductive and proliferative qualities of GIFT15 to generate large amounts of autologous inducible Bregs for personalized immunotherapy to treat MS patients.

References

1. Morell, P. & Quarles, R. The Myelin Sheath. American Society for Neurochemistry. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK27954. Accessed Februrary 24 2012.
2. Zieve, D. 2010. Multiple Sclerosis. A.D.A.M. Medical Encyclopedia. Retrieved from http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001747. Accessed Februrary 24 2012.
3. Correale, J., Balbuena Aguirre, M. E. & Farez, M. F. Sex-specific environmental influences affecting MS development. Clinical immunology (Orlando, Fla.) 4–9 (2013).doi:10.1016/j.clim.2013.02.006
4. Oksenberg, J. R., Baranzini, S. E., Sawcer, S. & Hauser, S. L. The genetics of multiple sclerosis: SNPs to pathways to pathogenesis. Nature reviews. Genetics 9, 516–26 (2008).
5. Olerup, O. & Hillert, J. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. Tissue antigens 38, 1–15 (1991).
6. Gebe, J. a, Swanson, E. & Kwok, W. W. HLA class II peptide-binding and autoimmunity. Tissue antigens 59, 78–87 (2002).
7. McFarland, H. F. & Martin, R. Multiple sclerosis: a complicated picture of autoimmunity. Nature immunology 8, 913–9 (2007).
8. Dutta, R. & Trapp, B. D. Pathogenesis of axonal and neuronal damage in multiple sclerosis. Neurology 68, S22–31; discussion S43–54 (2007).
9. Jeffery, D. R. Recent advances in treating multiple sclerosis: efficacy, risks and place in therapy. Therapeutic advances in chronic disease 4, 45–51 (2013).

10. Brown, J., William, L. & Coles, A. J. Alemtuzumab: evidence for its potential in relapsing-remitting multiple sclerosis. Drug design, development and therapy 7, 131–8 (2013).
11. Kala, M., Miravalle, A. & Vollmer, T. Recent insights into the mechanism of action of glatiramer acetate. Journal of neuroimmunology 235, 9–17 (2011).
12. Morbach, H. et al. Reference values for B cell subpopulations from infancy to adulthood. Journal of Translational Immunology. 162, 271-279 (2010)
13. Perez-Andres, M. et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. Cytometry Part B Clinical cytometry 78 Suppl 1, S47–S60 (2010).
14. Von Büdingen, H.-C., Bar-Or, A. & Zamvil, S. S. B cells in multiple sclerosis: connecting the dots. Current opinion in immunology 23, 713–20 (2011).
15. Bourdette, D. & Yadav, V. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. Current neurology and neuroscience reports 8, 417–8 (2008).
16. Shi, Y., Agematsu, K., Ochs, H. D. & Sugane, K. Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM. Clinical immunology Orlando Fla 108, 128–137 (2003).
17. Renaudineau, Y. et al. Autoreactive B cells and epigenetics. Clinical reviews in allergy immunology 39, 85–94 (2010).
18. Yanaba, K. et al. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity 28, 639–650 (2008).
19. Moore, K. W., Malefyt, R. D. W., Robert, L. & Garra, A. O. I Nterleukin -10 and the I Nterleukin -10. Molecular and Cellular Biology 1, 683–765 (2001).

20. Rafei, M. et al. A GMCSF and IL-15 fusokine leads to paradoxical immunosuppression in vivo via asymmetrical JAK/STAT signaling through the IL-15 receptor complex. Blood 109, 2234–42 (2007).
21. Waldmann, T. a & Tagaya, Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. Annual Review of Immunology 17, 19–49 (1999)
22. Tagaya, Y., Bamford, R. N., Defilippis, A. P. & Waldmann, T. A. IL-15 : A Pleiotropic Cytokine with Minireview Diverse Receptor / Signaling Pathways Whose Expression Is Controlled at Multiple Levels. 4, 329–336 (1996).
23. Deng, J. Galipeau, G. Reprogramming of B cells into regulatory cells with engineered fusokines. Infectious Disorders Drug Targets 3, 248-254 (2012).
24. Rafei, M. et al. A granulocyte-macrophage colony-stimulating factor and interleukin-15 fusokine induces a regulatory B cell population with immune suppressive properties. Nature Medicine 15, 1038–1045 (2009).
25. Duddy, M. et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. The Journal of Immunology 178, 6092–6099 (2007).
26. Niino, M., Hirotani, M., Miyazaki, Y. & Sasaki, H. Memory and naïve B-cell subsets in patients with multiple sclerosis. Neuroscience Letters 464, 74–78 (2009).
27. Xu, S. et al. IL-15 and dendritic cells induce proliferation of $CD4^{+}CD25^{+}$ regulatory T cells from peripheral blood. Immunology Letters 140, 59-67 (2011).