

B Cell Receptor Recognition of Glatiramer Acetate is Required for Efficacy through Antigen Presentation and Cytokine Production

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Abstract

Multiple Sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) leading to neuronal demyelination, lack of remyelination, and axonal loss. If left untreated, patients inevitably suffer from severe cognitive, psychological and physical disabilities. Although not yet approved, B cell depletion achieved with anti-CD20 monoclonal antibodies is the most effective therapy to-date in MS patients. As this therapeutic depletes immune cells potentially important in pathogen immunity, an immense need remains for highly efficacious therapeutics maintaining a favorable safety profile. Even amongst the emergence of new therapeutic options for patients with MS, the myelin basic protein mimetic, Copaxone (glatiramer acetate, GA), remains the most commonly prescribed drug in the United States. As specific B cell depletion appears to be the most effective therapy for MS patients, the goal of this study was to further elucidate the mechanism of action of GA on B lymphocytes. Our studies show that GA directly interacts with human and murine B cell receptors (BCR) inducing the activation of B lymphocytes and BCR recognition of GA is required for efficacy in an animal model of MS. GA loaded B lymphocytes resulted in IL-2 production from CD4⁺ T cells suggesting B lymphocytes serve as an antigen presentation source for GA. In fifty-percent of the MS patients tested, GA stimulation reduced baseline levels of the pro-inflammatory cytokines IL-6 and TNF α in purified B lymphocytes, while other cytokines were not consistently altered. Taken together, this data suggests that the mechanism of action of GA on B lymphocytes includes the presentation of GA to T lymphocytes in the context of an anti-inflammatory cytokine milieu. The results from this study provide a strong foundation for future exploration of optimal Copaxone responders or synergistic combination therapies with an improved risk: benefit ratio compared to currently approved therapeutics for MS.

Keywords: Copaxone; Glatiramer acetate; B cells; Immunology; Multiple sclerosis; EAE

Introduction

Multiple Sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) leading to neuronal demyelination and axonal loss resulting in cognitive, psychological and physical disabilities. Over the past decade, significant advances have been made in treatment options for patients. Newer therapies show improved efficacy compared to first-generation drugs, however they can be accompanied by serious and even fatal side effects [1-3]. Better understanding of the mechanism of action of therapeutics may lead to highly efficacious therapies with a favorable safety profile.

The MS-like disease animal model, experimental autoimmune encephalomyelitis (EAE), has provided invaluable information about the role of encephalitogenic T cells, antigen presentation, demyelination, and remyelination [4-7]. Although numerous T cell directed therapies significantly reduce disease severity in this model [8,9], T cell depletion with anti-CD3 and anti-CD4 monoclonal antibodies failed in clinical trials for MS [10,11]. Depletion of peripheral B cells with anti-CD20 monoclonal antibodies in EAE enhances or inhibits disease severity dependent upon the time of dosing and the specific EAE model used. In the MOG 35-55 model, B cell depletion prior to disease onset enhances disease severity, while therapeutic depletion significantly decreases disease severity [12]. In the rMOG 1-125 model, B cell depletion prior to disease onset and therapeutic dosing significantly reduces disease severity [13]. These data suggest that the role of B cells is dependent on the model, the time of dosing, and suggests protective and pathogenic functions. Contrary to the murine studies, specific B cell depletion with anti-CD20 monoclonal antibodies in humans with MS is the most effective treatment to-date [14-24]. These therapeutics, rituximab, ofatumumab, and ocrelizumab, remain unapproved and have long-term safety concerns. One of the safest therapies for MS patients is Copaxone

(glatiramer acetate, GA). The mechanism of action of Copaxone has been studied extensively and a wide range of activities have been reported including CNS protection, as well as immunomodulatory effects on T cells, monocytes, dendritic cells, and B cells [25-53]. The majority of the published effects of GA focus on its ability to modulate T cell responses through innate immune cells. As B cells appear to be central to disease pathogenesis in MS, further exploration of the effects of GA in B lymphocytes is warranted.

In humans, the only data indicative of a direct effect of Copaxone on B lymphocytes is that essentially all patients on Copaxone form antibodies to the drug [54-59]. This suggests that Copaxone directly interacts with the B cell receptor (BCR) on B lymphocytes. These antibodies are non-neutralizing and do not alter the efficacy of the drug. As BCR engagement alone is insufficient to induce class-switching to other antibody isotypes, the data suggests that either patients have cross-reactive antibodies prior to Copaxone treatment or the environmental milieu in the presence of Copaxone is sufficient to provide the second signal required for high-affinity antibody production. The former is unlikely as patient samples obtained prior to Copaxone treatment

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had undetectable levels of antibody to the drug [57,59]. Therefore, it is probable that the class-switched Copaxone antibodies observed in MS patients result from direct binding to the BCR plus an additional second signal acquired *in vivo*. In mice, it has been shown that B lymphocytes transferred from GA treated mice into EAE recipients are sufficient to reduce disease severity [33,35]. One study reported that CD19⁺CD5⁺ B cells transfer the GA effect with an increased percentage of these cells expressing anti-inflammatory IL-4 and IL-10. The other study showed a reduction in pro-inflammatory cytokine production and an increase in anti-inflammatory IL-10 from spleen and lymph node MOG 35-55 specific T cells after adoptive transfer of CD43-B cells from GA treated mice. Although these functional outcomes support a role for B cells in GA mediated efficacy, it remains unknown if this is the result of a direct or indirect interaction.

The goal of this study was to further elucidate the effects of Copaxone on B lymphocytes. We found that the protective effect of GA in EAE is mediated through an interaction with the BCR and likely involves multiple mechanisms including antigen presentation and cytokine production. GA also bound at least in-part to the BCR on B lymphocytes from MS patients. Interestingly, in half of the MS patients, GA reduced baseline levels of the pro-inflammatory cytokines IL-6 and TNF α . This data, in combination with previous reports showing an alteration of the cytokine profiles of GA specific B cells, suggest that the mechanism of action of GA on B lymphocytes includes the presentation of GA to T lymphocytes, and cytokine production similar to what has been reported with other innate immune cells [33,35]. Taken together, these data provide a strong foundation for future exploration of optimal GA responders or synergistic combination therapies with an improved risk: benefit ratio compared to currently approved therapeutics for MS.

Materials and Methods

Mice

All animal studies performed were in accordance with and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado Denver. C57BL/6 animals were purchased from Taconic (B6F) or Jackson Labs (000664). The transgenic or knock-out mice were purchased from Jackson labs with the following catalog information. C57BL/6-Tg(TcraTcrb)1100Mjb/J TCR knock-in (003831), C7BL/6-TG(TcraTcrb)425Cbn/J TCR knock-in (004194), C7BL/6 129S2-H2 diab1-ea/J MHC class II knock-out (003584), C57BL/6 129P2-B2m tm1Unc/J MHC class I knock-out (002087), and C57BL/6-Tg(IghelMD4)4Ccg/J MD4 knock-in mice (002595).

Human specimens

All human samples obtained were in accordance with and approved by the Colorado Multiple Institutional Review Board (COMIRB).

Complete medium and conjugation of reagents

Complete medium is composed of RPMI media (Hyclone, SH30027.01) containing 10% fetal bovine serum (Hyclone, SH30071.03), 5 mL penicillin-streptomycin (Hyclone, SV30010) and 5 mL non-essential amino acids (Hyclone, SH30238.01). Antigen presentation medium is complete medium plus the addition of 2-Betamercaptoethanol. Pharmaceutical grade glatiramer acetate or anti-IgM (company) was conjugated to DyLight 488 (Thermo-Pierce 53024) or biotin (Thermo Pierce, 21425), according to the manufacturer's protocols.

GA binding experiments

Spleens from the indicated mice were homogenized, pelleted, red

cell depleted with ACK lysis buffer (Bio-Whittaker, 10-548-E) and suspended in complete media. Splenocytes were plated in 96 well, round bottom plates at 200,000 cells per well in 100 μ L complete media. Each genotype was plated in a separate column. Cells were incubated in triplicate for 30 minutes with either 488-conjugated glatiramer acetate (1:1000 dilution of 1 mg/ml stock in PBS) (TEVA, 242903212), or 488 alone. After incubation, the cells were washed extensively and incubated for 30 minutes with the following antibodies specific for mouse. V450 conjugated anti-CD4 (1:200) (BD Horizon, 560468), APC conjugated anti-CD8 (1:200) (BD Pharmingen, 553035) and PerCP CY 5.5 conjugated anti-CD11c (1:200) (BD Pharmingen, 560584), or V450 conjugated anti-F4/80 (1:100) (eBiosciences, 48-4801-80), APC conjugated anti-NK1.1 (1:200) (BD Pharmingen, 561117) and PerCP CY 5.5 conjugated anti-B220/CD45R (1:400) (BD Pharmingen, 561101). After the incubation period, the cells were washed extensively and fixed for 15 minutes at room temperature using 2% paraformaldehyde solution (Macron Chemicals, H121-05). The fixation was washed off followed by flow cytometric acquisition and analysis using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

GA competition assay: Purified B lymphocytes from wild-type mice were incubated with 10 μ g/ml of fluorochrome labeled GA for 30 minutes or incubated with unlabeled GA for 30 minutes, washed and incubated with labeled GA for 30 minutes. The cells were washed extensively and fixed in 2% paraformaldehyde solution (Macron Chemicals, H121-05). The percent of GA^{hi} B cells was determined by flow cytometric acquisition and analysis using BD™ LSR II Flow Cytometer (BD Biosciences) and FlowJo (TreeStar Inc.).

Ex vivo GA binding post GA treatment: Wild-type C57BL/6 mice (three mice per treatment group) were treated daily with 100 μ L injections of 200 μ g/mL glatiramer acetate (TEVA Neuroscience, NDC 68546-317-30) in PBS (Corning Cellgro, 21-030-CV) or PBS alone s.c. The spleens were removed and processed into single cell suspensions as described above. Cells were plated in a 96 well, round bottom plate at 250,000 cells per well in 100 μ L complete medium. Cells were then incubated in duplicate with 488-conjugated glatiramer acetate (1:1000 dilution of 1 mg/ml stock in PBS) (Thermo-Pierce 53024), or the 488 fluorochrome alone in the dark for 30 minutes at room temperature. The cells were washed extensively and incubated with two individual stain mixes for 30 minutes at room temperature of either APC conjugated anti-mouse NK 1.1 (1:200) (BD Pharmingen, 561101), PerCP CY 5.5 conjugated anti-mouse CD11c (1:200) (BD Pharmingen, 560584) and V450 conjugated anti-mouse F4/80 (1:100) (eBiosciences, 48-4801-80), or APC conjugated anti-mouse CD8 (1:200) (BD Pharmingen, 553035), V450 conjugated anti-mouse CD4 (1:200) (BD Horizon, 560468) and PerCP CY 5.5 conjugated anti-mouse B220/CD45R (1:400) (BD Pharmingen, 561101). Cells were then washed and fixed for 15 minutes at room temperature in a 2% paraformaldehyde solution (Macron Chemicals, H121-05). Cells were then washed and suspended in PBS. The data was acquired by flow cytometry using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

Intracellular and extracellular GA binding: Spleens were removed from the indicated mice and processed into single cell suspensions as described above. B cells were isolated using the Miltenyi CD43- B cell isolation kit (Miltenyi Biotec, 130-095-813). Cells were plated in a 96 well round bottom plate at 500,000 cells per well in 100 μ L complete medium. Cells were incubated with biotin conjugated glatiramer acetate (1:500 dilution of 1 mg/ml stock) or 10 μ g/ml anti-IgM (Southern

Biotech, 1023-01) for the indicated time-points and resuspended in 100 μ L of 488 conjugated streptavidin (Life Technologies, S11223) at a 1:500 dilution in PBS (Corning Cellgro, 21-030-CV) containing 0.5% fetal bovine serum for 30 minutes at room temperature. The cells were fixed in 100 μ L 2% paraformaldehyde solution for 10 minutes at room temperature. After centrifugation, the cells were then washed in saponin cell permeabilization buffer (eBiosciences, 00-8333-56). The cells were then suspended in a solution of APC-conjugated streptavidin (BD Pharmingen, 554067) in saponin cell permeabilization buffer (eBiosciences, 00-8333-56). The cells were incubated in the dark for 30 minutes at room temperature followed by extensive washing and fixed again in 2% paraformaldehyde solution for 10 minutes at room temperature. The cells were again washed extensively and resuspended in PBS + 0.5% FBS. Data was acquired by flow cytometry using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

GA presentation by B cells to T cells

Spleens were removed from two wild-type C57BL/6 mice and processed as described above to obtain a single cell suspension. B cells were isolated using Miltenyi CD43- B cell isolation kit (Miltenyi Biotec, 130-095-813). Purified B cells were incubated with the indicated antigen for 18-20 hours in antigen presentation media, washed and incubated with freshly isolated splenic CD4⁺ T cells obtained from an additional wild-type mouse using the Miltenyi CD4⁺ T cell isolation kit II (Miltenyi Biotec, cat #) in a 2:1 ratio of 400,000 B cells to 200,000 CD4⁺ T cells per condition in antigen presentation media. Cells were then incubated for 48 hours at 37°C with 5% CO₂. At the 48-hour time point, the plate was centrifuged at 300 \times g for 5 minutes and 100 μ L of supernatant was transferred to a 96 well, round bottom plate and frozen at -20°C. Supernatants were later thawed and analyzed for IL-2 expression with Meso Scale Discovery Cytokine Panel 6 MULTI-SPOT™ 96 well plate (Meso Scale Discovery, K152AHB-2).

Intracellular phosphotyrosine staining and upregulation of activation markers on B cells

Intracellular phosphotyrosine staining: B cells were purified from C57BL/6 splenocytes according to the manufacturers protocol (Miltenyi Biotec, 130-095-813). B Cells were plated in two 96 well, round bottom plates at 200,000 cells per well in 100 μ L complete medium. Cells were then incubated with glatiramer acetate (1:1000 concentration) (TEVA, 242903212) conjugated to a 488 fluorochrome (Thermo-Pierce, 53024), anti-mouse IgM at 10 μ g/mL (Southern Biotech, 1023-01) or PBS in duplicate for 1, 5, and 10 minutes. At the end of the indicated time period, cells were immediately fixed in 2% paraformaldehyde for 10 minutes at room temperature, followed by washing and intracellular staining with an anti-phosphotyrosine antibody (Millipore, 16-103). Flow cytometric data was acquired using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

Upregulation of activation markers: B cells were purified from C57BL/6 splenocytes according to the manufacturers protocol (Miltenyi Biotec, 130-095-813). B Cells were plated in two 96 well, round bottom plates at 200,000 cells per well in 100 μ L complete medium. Cells were then incubated with glatiramer acetate (1:1000 concentration) (TEVA, 242903212) conjugated to a 488 fluorochrome (Thermo-Pierce, 53024), anti-mouse IgM at 10 μ g/mL (Southern Biotech, 1023-01) or PBS in duplicate for 24 or 48 hours at 37°C and 5% CO₂. After 24 hours, the cells in one of the plates were incubated with PerCP CY 5.5 conjugated anti-mouse CD69 (at 1:200) (BD Pharmingen, 561931) and APC

conjugated anti-mouse B220/CD45R (at 1:400) (BD Pharmingen, 561880). Antibodies were incubated in the dark for 40 minutes at room temperature washed and fixed in 2% paraformaldehyde solution (Macron Chemicals, H121-05) for 10 minutes. After 48 hours, the cells in the other plate were incubated with V450 conjugated anti-mouse MHC class II (eBiosciences, 48-5320-80), PE conjugated anti-mouse CD86 (eBiosciences, 12-0862-82) and APC conjugated anti-mouse B220/CD45R (at 1:400). Flow cytometric data was acquired using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

Experimental autoimmune encephalomyelitis

EAE induction and scoring: Mice were anesthetized via CO₂ narcosis and administered two 100 μ L subcutaneous injections, one at the neck and one at the base of the tail, of 2 mg/mL myelin-oligodendrocyte glycoprotein 35-55 emulsified in Complete Freund's Adjuvant from an EAE induction kit (Hooke Labs, EK-0114) EAE mice were also injected i.p. with 100 μ L 375 ng/mL pertussis toxin, also included in the induction kit. A second i.p dose of 100 μ L 375 ng/mL pertussis toxin was administered to the animals 18-20 hours post the first dose. On day 2, post EAE induction, mice were inspected and scored according to a numerical range from 0-5. Mice were then scored every other day until the completion of the study. The scoring range for EAE ranges from 0, no impairment, to 5, complete paralysis/moribund. The scoring system used is as follows, 0.5=lack of tail tone, 1=complete loss of tail tone, 1.5=complete loss of tail tone and hind limb weakness, 2=complete loss of tail tone and hind limb weakness, 2.5=complete loss of tail tone and either incomplete paralysis of both hind limbs or complete paralysis of one hind limb, 3=complete hind limb paralysis, 3.5=complete hind limb paralysis and impairment in one forelimb, 4=complete hind limb paralysis and impairment in both forelimbs or partial paralysis in one fore limb, 4.5=complete hind limb paralysis and paralysis in one forelimb, 5=complete hind and front limb paralysis, or moribund. Animals are routinely euthanized if a lesion from the immunization begins to bore or if the animals reach score between 4 and 4.5. It is very rare that the disease is this severe and this was not observed in our studies. After a score of 2 is seen in any group of mice a gelatinous food source is placed on the bottom of the cage and animals are monitored for full bladders. Any animal with a full bladder has it expressed manually.

In vivo GA treatment and cellular transfers: Three wild-type C57BL/6, BCR-HEL tg or MHC Class I deficient mice were treated for 14 days with daily subcutaneous injections of 100 μ L 2 mg/mL glatiramer acetate (TEVA, 242903212) diluted in PBS (Corning Cellgro, 21-030-CV) or a control of 100 μ L PBS. Spleens and lymph nodes were removed from the treated animals and CD43-B cells (Miltenyi Biotec, 130-049-801) or CD4⁺ T cells (Miltenyi Biotec, 130-095-458) were isolated by negative selection. These cells (5 \times 10⁶ purified B or T cells/mouse) were harvested and injected into EAE recipients on the day of EAE induction. Animals that did not receive the transferred cells were treated s.c. daily with 100 μ L of 2 mg/ml GA in PBS, or PBS alone.

Organ harvest and processing: Upon completion of the study, mice were anesthetized using isoflurane (Piramal Healthcare, 66794-013-25) and perfused with 0.1 M phosphate buffered saline for five minutes or until the blood visibly cleared from the liver. The brain, spinal cord, spleen and both inguinal lymph nodes were removed and placed into complete media and homogenized using a dounce homogenizer. Mononuclear cells were isolated from the brain and spinal cord using a Percoll gradient (Sigma-Aldrich, P4937) gradient. Cells were then plated in a 96 well round bottom plate at 200,000

cells per well in 100 μ L RPMI medium solution. Each tissue type was plated in two columns and stained with one of two antibody mixes. All wells were first incubated with glatiramer acetate conjugated to a 488 fluorochrome (Thermo-Pierce, 53024), at a concentration of 1:1000. Glatiramer acetate-488 was incubated for 30 minutes at room temperature, in the dark. The plate was then centrifuged at 300 \times g for minutes and the supernatant was removed. Cells were then labeled with either APC conjugated anti-mouse NK 1.1 (1:200) (BD Pharmingen, 561101), PerCP CY 5.5 conjugated anti-mouse CD11c (1:200) (BD Pharmingen, 560584) and V450 conjugated anti-mouse F4/80 (1:100) (eBiosciences, 48-4801-80), APC conjugated anti-mouse CD8 (1:200) (BD Pharmingen, 553035), V450 conjugated anti-mouse CD4 (1:200) (BD Horizon, 560468) and PerCP CY 5.5 conjugated anti-mouse B220/CD45R (1:400) (BD Pharmingen, 561101). Antibodies were incubated for 30 minutes at room temperature, in the dark and then centrifuged to remove supernatants. Cells were then fixed in a 2% paraformaldehyde solution (Macron Chemicals, H121-05). Cells were analyzed via flow cytometry using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

Human GA experiments

Blood was obtained from treatment naïve MS patients using BD Vacutainer Cell Preparation Tubes with 1 mL 0.1 M Sodium Citrate solution (BD Biosciences, 362761). The blood was separated into plasma and peripheral blood mononuclear cells via centrifugation at 1500 \times g for twenty minutes with no brake. Cells were removed from gradient layer and washed three times with 20 mL PBS (Corning Cellgro, 21-030-CV). Cell suspensions were centrifuged at 300 \times g for 5 minutes during washes. Cells were suspended in RPMI medium (Hyclone, SH30027.01) containing 15% fetal bovine serum (FBS) (Hyclone, SH30071.03), 10% dimethyl sulfoxide (Fischer Bioreagents, BP231-1) and 5 mL penicillin-streptomycin (Hyclone, SV30010). Cells were stored at -80°C until enough samples were processed to run the experiment. Once all ten donors had been processed, three 1 mL aliquots of cells from each donor were thawed at room temperature and diluted in RPMI medium containing 10% fetal bovine serum, 5 mL penicillin-streptomycin and 5 mL non-essential amino acids (Hyclone, SH30238.01) at a ratio of 1:5 for a total volume of 25 mL. The cells were then centrifuged at 300 \times g for 5 minutes. Supernatant was removed and the cells were suspended in 550 μ L DPBS containing 0.5% fetal bovine serum. Cells were then plated on a 96 well, round bottom plate at 100 μ L per well, 5 wells per column. In the first round of staining, glatiramer acetate (TEVA, 242903212) conjugated to a 488 fluorochrome (Thermo-Pierce 53024), at a concentration of 1:1000, was added to two testing rows. A solution of the 488 fluorochrome alone in PBS, at a concentration of 1:1000, was added to the other two testing rows. Antibodies were incubated in the dark for 60 minutes at 37°C. The plate was then centrifuged at 300 \times g for 5 minutes and the supernatant was removed. In the second round of staining, two antibody mixes, diluted in DPBS-FBS solution, were made to stain for cell surface markers. Cells were resuspended with either V450 conjugated anti-human CD3 (1 μ L per well) (BD Horizon, 560366), APC conjugated anti-human CD19 (2.5 μ L per well) (product ID) and PerCP CY 5.5 anti-human CD20 (5 μ L per well) (Becton-Dickinson, 340955), or PE conjugated anti-human CD56 (2.5 μ L per well) (BD Pharmingen, 561903) and APC conjugated anti-human CD14 (2.5 μ L per well) (BD Pharmingen, 561708). Cells were resuspended in 100 μ L antibody mixes and incubated in the dark for 60 minutes at room temperature. Cells were then centrifuged at 300 \times g for 5 minutes and supernatants were removed. Cells were then fixed in a 2% paraformaldehyde solution (Macron Chemicals, H121-05) Data

was acquired via flow cytometry using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

Competition assay: Blood was obtained from three treatment naïve MS donors using BD Vacutainer Cell Preparation Tubes with 1 mL 0.1 M Sodium Citrate solution (BD Biosciences, 362761) and processed into PBMCs following the manufacturer's protocol. B cells were purified using Miltenyi B cell Isolation kit II (Miltenyi Biotec, 130-091-151) according to the manufacturer's protocol. B cells were plated in a 96 well, round bottom plate at 100,000 cells per well in 100 μ L DPBS-FBS solution. Glatiramer acetate (TEVA, 242903212) conjugated to a 488 fluorochrome (Thermo-Pierce 53024), at a concentration of 1:1000, or V450 conjugated anti-human IgG (BD Pharmingen, 555785), at a concentration of 20 μ g/mL, was incubated with the cells for 30 minutes at room temperature. The cells were then washed and the reciprocal was added to the B cells for 30 minutes at room temperature. Cells were then fixed in a 2% paraformaldehyde solution (Macron Chemicals, H121-05). Data was acquired via flow cytometry using BD™ LSR II Flow Cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo (TreeStar Inc.).

GA stimulated cytokine production from B cells: Blood was obtained from six MS patients using BD Vacutainer Cell Preparation Tubes with 1 mL 0.1 M Sodium Citrate solution (BD Biosciences, 362761) and processed into PBMCs according to the manufacturer's protocol. B cells were then purified by negative cell selection using Miltenyi B cell Isolation kit II (Miltenyi Biotec, 130-091-151). Isolated B cells were then plated in a 96 well, round bottom plate between at 100,000 cells per well in 200 μ L medium. The positive control was B cells were incubated glatiramer acetate at a 1:1000 dilution of 1 mg/ml stock (TEVA, 242903212), glatiramer acetate at a 1:1000 dilution of 1 mg/ml stock and anti-human CD40 at 0.25 μ g/mL or anti-human IgM at 20 μ g/mL (Southern Biotech, 2022-08) and anti-human CD40 at 0.25 μ g/mL (eBiosciences, 14-0409-82). The final well was left unstimulated. Cells were placed in an incubator for 72 hours at 37 degrees and 5% CO₂. Every 24 hours, cells 65 μ L of supernatant was removed, placed in a 96 well plate and frozen at -20°C. After all three time points were collected, supernatants were thawed and analyzed with Meso Scale Discovery human proinflammatory-7 MULTI-SPOT® 96 well (Meso Scale Discovery, N75008A-1) and Cytokine panel 4 MULTI-SPOT® 96 well (Meso Scale Discovery, N45018A-1) plates.

Results

GA binds to the B cell receptor, and *in vivo* administration of GA in a non-inflammatory environment does not expand GA specific B lymphocytes

Recognition of GA by B lymphocytes could occur through the B cell receptor (BCR), other cell surface molecules such as major histocompatibility complex (MHC), or by other specific or non-specific mechanisms. To evaluate if GA binds to the BCR or MHC on B cells, fluorochrome conjugated GA was incubated with purified B lymphocytes from wild-type mice, MHC class I or Class II deficient mice, or mice with a BCR transgene specific for hen egg lysozyme (BCR-HEL tg). B lymphocytes from mice with a CD4 or CD8 TCR transgene specific for ovalbumin (OVA) were used as controls. A small proportion of GA negative cells was observed as well as two distinct GA positive B cell populations, termed GA^{hi} and GA^{lo} (Figures 1A and 1B). Seventy to ninety percent of the GA^{hi} population was abrogated on B lymphocytes obtained from BCR-HEL tg animals, suggesting that most of the observed GA positivity was due to a specific interaction

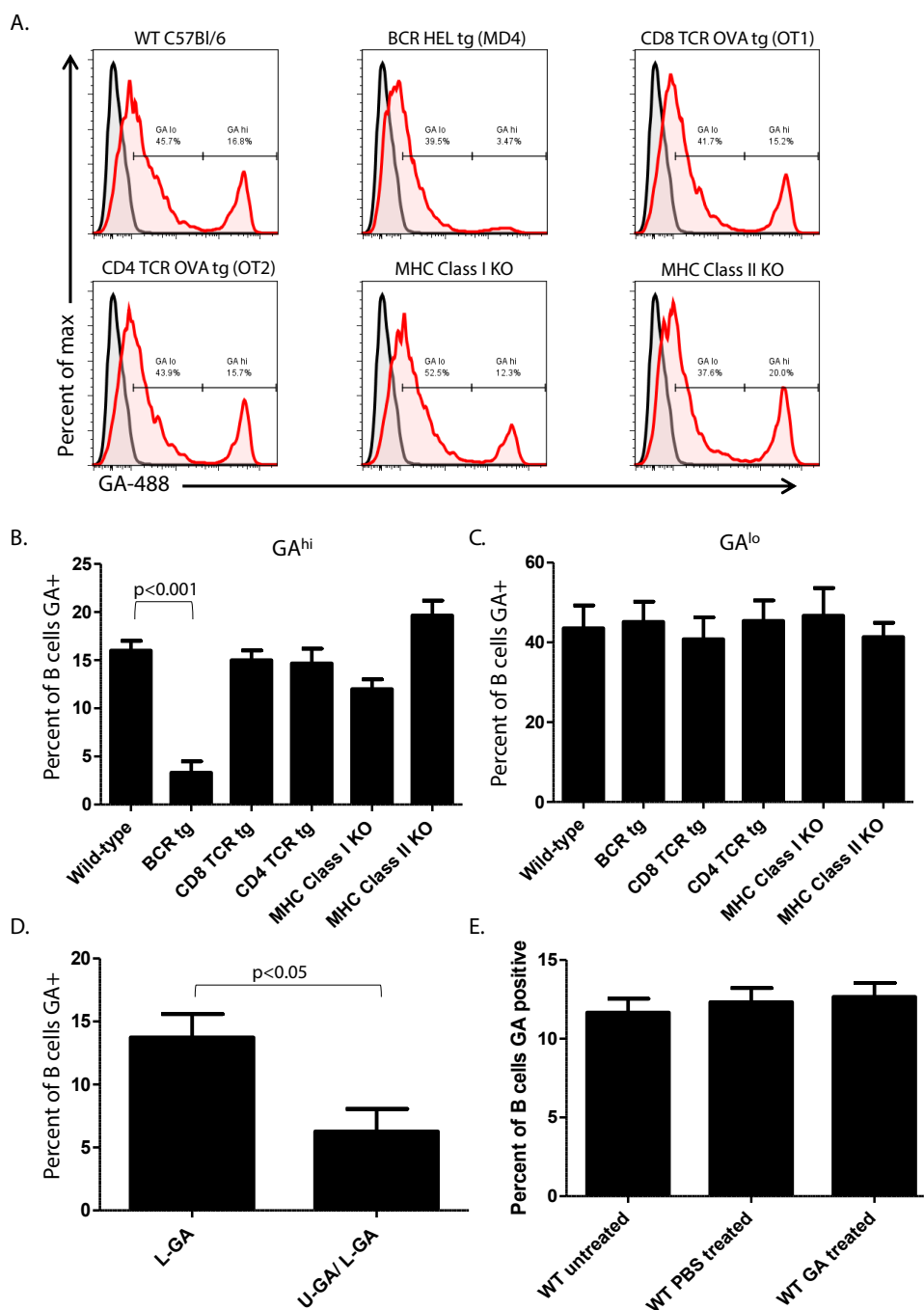


Figure 1: GA binds to the B cell receptor, and *in vivo* administration of GA in a non-inflammatory environment does not expand GA specific B lymphocytes. Single cell suspensions of splenocytes from the indicated mice were incubated with 488 conjugated GA and stained with antibodies specific for T cells, B cells, macrophages, and NK cells. The percent of GA⁺ immune cell populations was determined by flow cytometric acquisition and analysis. Shown is the data generated for B lymphocytes. (A) GA non-specifically associates with B cells as shown by the GA^{lo} gated population and specifically binds to the B cell receptor as shown by reduced binding in the GA^{hi} population on BCR-HEL tg B lymphocytes. Black lines represent the staining profile of 488 alone, while the red lines represent the staining profile with 488-GA. Data is representative of three individual experiments. (B) The reduction in the GA^{hi} population on B lymphocytes from BCR-HEL tg animals is statistically significant. The reduction in the proportion of GA^{hi} B cells from MHC Class I KO animals and the increased proportion of GA^{hi} B cells from MHC Class II KO animals was not statistically significant. The graph shows the average proportion of GA^{hi} B cells \pm SD from three separate experiments. Statistical analysis was performed using Kruskal-Wallis with Dunnett post-test. (C) The proportion of GA^{lo} B lymphocytes is not statistically different in all mouse strains tested. The graph shows the average proportion of GA^{lo} B cells \pm SD from three separate experiments. Statistical analysis was performed using Kruskal-Wallis with Dunnett post-test. (D) Splenic B cells from wild-type mice were either incubated with labeled GA (L-GA), or unlabeled GA (U-GA) followed by labeled GA. Shown is the percent of B cells GA^{hi}. (E) *In vivo* administration of GA does not expand GA⁺ B lymphocytes in wild-type mice. The graph shows the average proportion of GA⁺ B lymphocytes \pm SD from three mice per group. Data is representative of three experiments. Statistical analysis was performed using Kruskal-Wallis with Dunnett post-test.

between GA and the BCR. The remaining GA^{hi} B cells appears to represent an interaction between GA and MHC Class I, as B cells obtained from Class I deficient mice consistently bound 10-30% less GA than wild-type mice, however this reduction was not statistically significant. B lymphocytes deficient in MHC Class II appeared to have a consistent but insignificant increase in the GA^{hi} population. This may

be due to the reported increased expression of BCR's on MHC Class II deficient B lymphocytes (reference). Therefore, it is also possible that GA binds to MHC Class II, however this data may have been masked due to the increased expression of BCR's bound to GA. It is important to note that GA also bound to a small percentage of monocytes (5-15%) and CD4⁺ T cells (3-10%) but very few, if any, CD8⁺ T cells or natural killer cells (data not shown). This binding data supports others work showing effects of GA on various immune cell populations [25,27,28,31,36,37,39]. An interesting finding is that a shift in the negative population not observed with the fluorochrome alone, termed GA^{lo}, was observed when B lymphocytes were incubated with GA (Figures 1A and 1C). The proportion of GA^{lo} B lymphocytes was not altered in any of the mouse strains tested, suggesting that this is either an artifact of the assay conditions, a large proportion of B lymphocytes may bind GA non-specifically, or it is internalized by active or passive mechanisms. This same phenomenon was also observed in human B cells (Figures 6A and 6B) and the significance of this low level of GA positivity remains unknown. The maximum percentage of GA⁺ B cells occurred at a concentration of 10 µg/ml of GA. To determine if this amount of drug saturates the BCR, a competition assay was performed. B lymphocytes from wild-type animals were incubated with 10 µg/ml of unlabeled GA, followed by incubation with 10 µg/ml of labeled GA. There was approximately 40% less GA⁺ B cells, when pre-incubated with unlabeled GA. This suggests that although the maximum amount of GA⁺ B cells was achieved at this concentration, it is not sufficient to saturate all BCR's (Figure 1D). As the B lymphocytes that bound high levels of GA mostly occurred through the BCR, we hypothesized that *in vivo* administration of GA may expand GA specific B lymphocytes. Wild-type animals were administered 200 µg GA s.c. for 14 days and the percent of GA^{hi} splenic B lymphocytes was determined by flow cytometric acquisition and analysis. *In vivo* administration of GA did not increase the proportion of GA specific splenic B lymphocytes (Figure 1E). As GA is derived from myelin amino acids, it is possible that in a non-inflammatory environment, GA specific B lymphocytes traffic to or expand in the brain or spinal cord. In non-diseased animals no change was observed in the percent of GA specific cells in the brain or spinal cord in GA treated mice compared to animals that received PBS (Figure 5B).

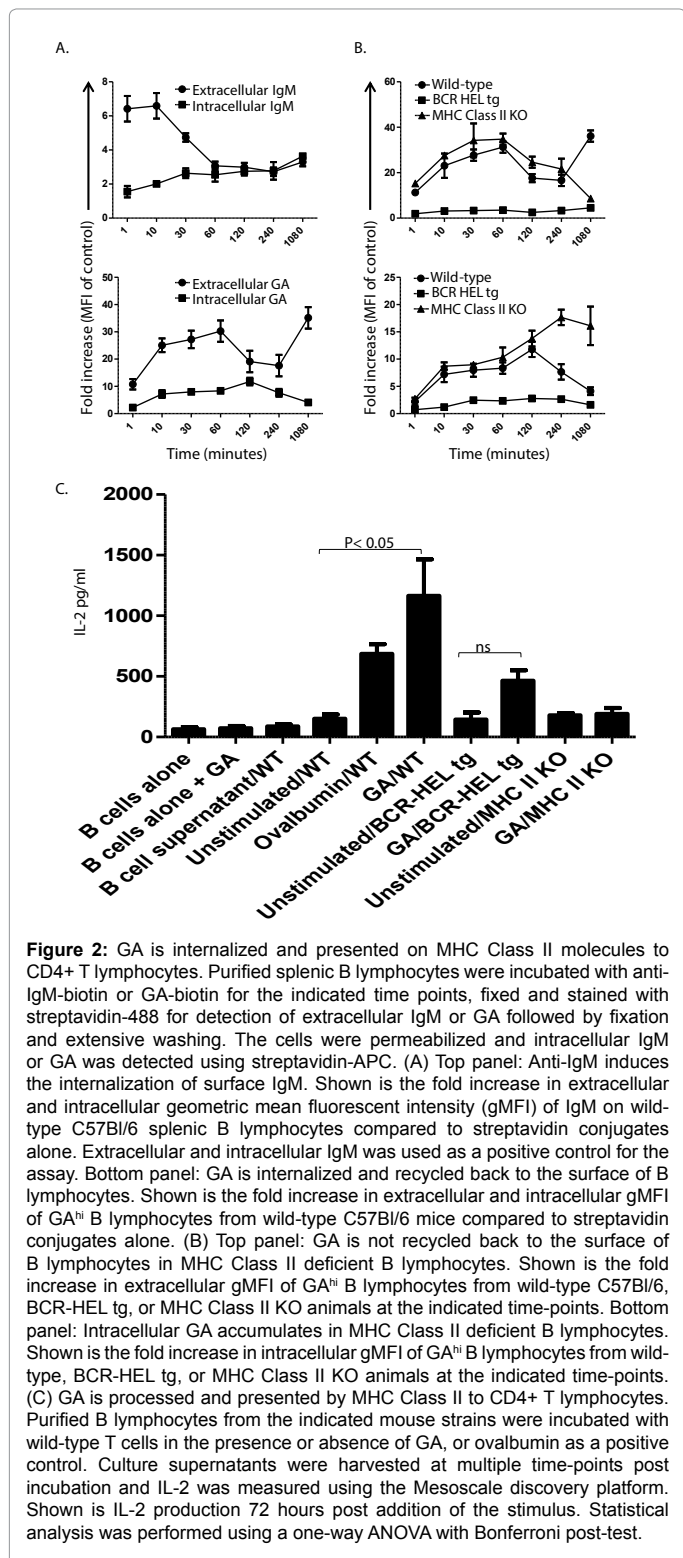
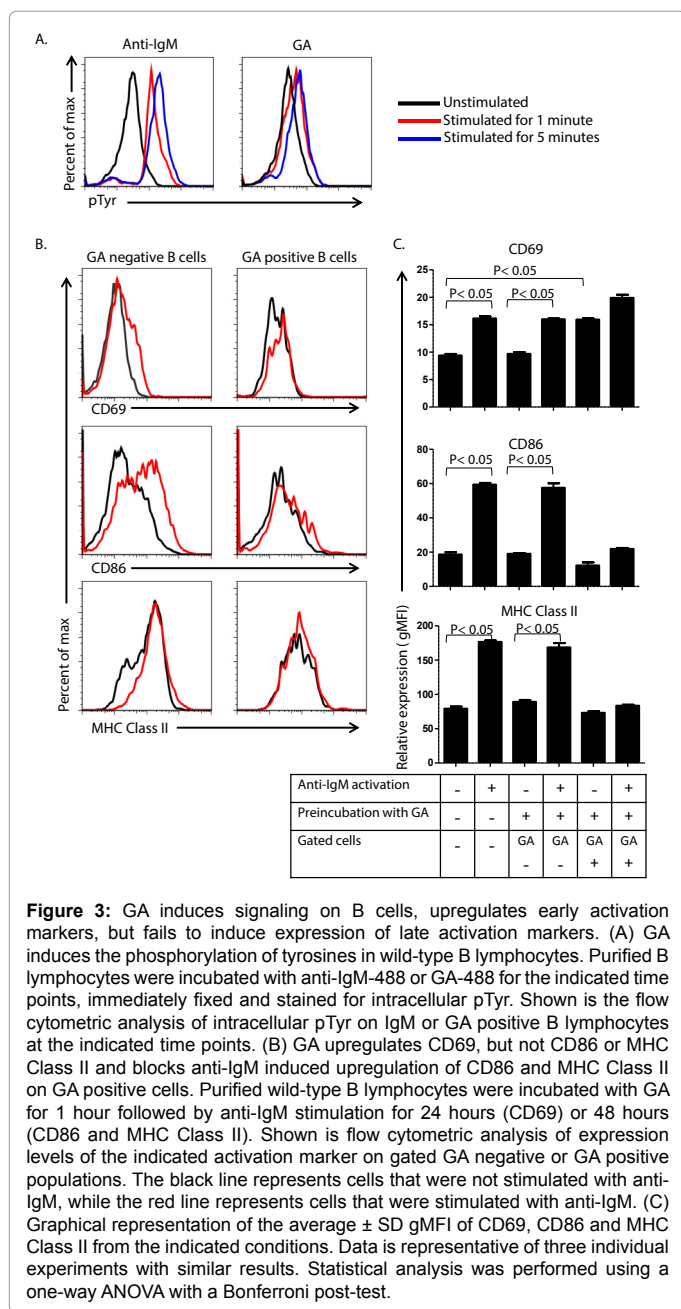


Figure 2: GA is internalized and presented on MHC Class II molecules to CD4⁺ T lymphocytes. Purified splenic B lymphocytes were incubated with anti-IgM-biotin or GA-biotin for the indicated time points, fixed and stained with streptavidin-488 for detection of extracellular IgM or GA followed by fixation and extensive washing. The cells were permeabilized and intracellular IgM or GA was detected using streptavidin-APC. (A) Top panel: Anti-IgM induces the internalization of surface IgM. Shown is the fold increase in extracellular and intracellular geometric mean fluorescent intensity (gMFI) of IgM on wild-type C57Bl/6 splenic B lymphocytes compared to streptavidin conjugates alone. Extracellular and intracellular IgM was used as a positive control for the assay. Bottom panel: GA is internalized and recycled back to the surface of B lymphocytes. Shown is the fold increase in extracellular and intracellular gMFI of GA^{hi} B lymphocytes from wild-type C57Bl/6 mice compared to streptavidin conjugates alone. (B) Top panel: GA is not recycled back to the surface of B lymphocytes in MHC Class II deficient B lymphocytes. Shown is the fold increase in extracellular gMFI of GA^{hi} B lymphocytes from wild-type C57Bl/6, BCR-HEL tg, or MHC Class II KO animals at the indicated time-points. Bottom panel: Intracellular GA accumulates in MHC Class II deficient B lymphocytes. Shown is the fold increase in intracellular gMFI of GA^{hi} B lymphocytes from wild-type, BCR-HEL tg, or MHC Class II KO animals at the indicated time-points. (C) GA is processed and presented by MHC Class II to CD4⁺ T lymphocytes. Purified B lymphocytes from the indicated mouse strains were incubated with wild-type T cells in the presence or absence of GA, or ovalbumin as a positive control. Culture supernatants were harvested at multiple time-points post incubation and IL-2 was measured using the Mesoscale discovery platform. Shown is IL-2 production 72 hours post addition of the stimulus. Statistical analysis was performed using a one-way ANOVA with Bonferroni post-test.

GA is internalized and presented on MHC Class II molecules to CD4⁺ T lymphocytes

Antigen presentation (APC) by B lymphocytes requires the ability of the cells to uptake extracellular antigens through cell surface molecules or phagocytic mechanisms. The antigens are internally processed and presented on MHC Class II molecules to CD4⁺ T lymphocytes. Since the majority of specific GA binding was to the BCR, we hypothesized that GA may be internalized, processed and presented on MHC Class II to CD4⁺ T cells. Initially, this series of experiments was attempted in the presence of internalization and recycling inhibitors including Latrunculin-B, Nystatin, Chlorpromazine, Methyl-β-cyclodextrin and genistein. At doses that did not induce cell death, these inhibitors only worked partially, even on the positive control IgM, making analysis of the data difficult. As an alternative, the kinetics and internalization of GA was evaluated by incubating biotinylated GA with purified splenic B cells for the indicated time-points. At the end of the incubation period, cells were stained extracellularly with avidin-488, fixed, and stained intracellularly with avidin-APC. Therefore, the intensity of 488 represented extracellular GA, while the intensity of APC represented intracellular GA. Biotinylated anti-IgM was used as a positive control, as it is actively internalized through the BCR (Figure 2A top panel). GA rapidly bound to the extracellular surface of B cells, reaching its peak



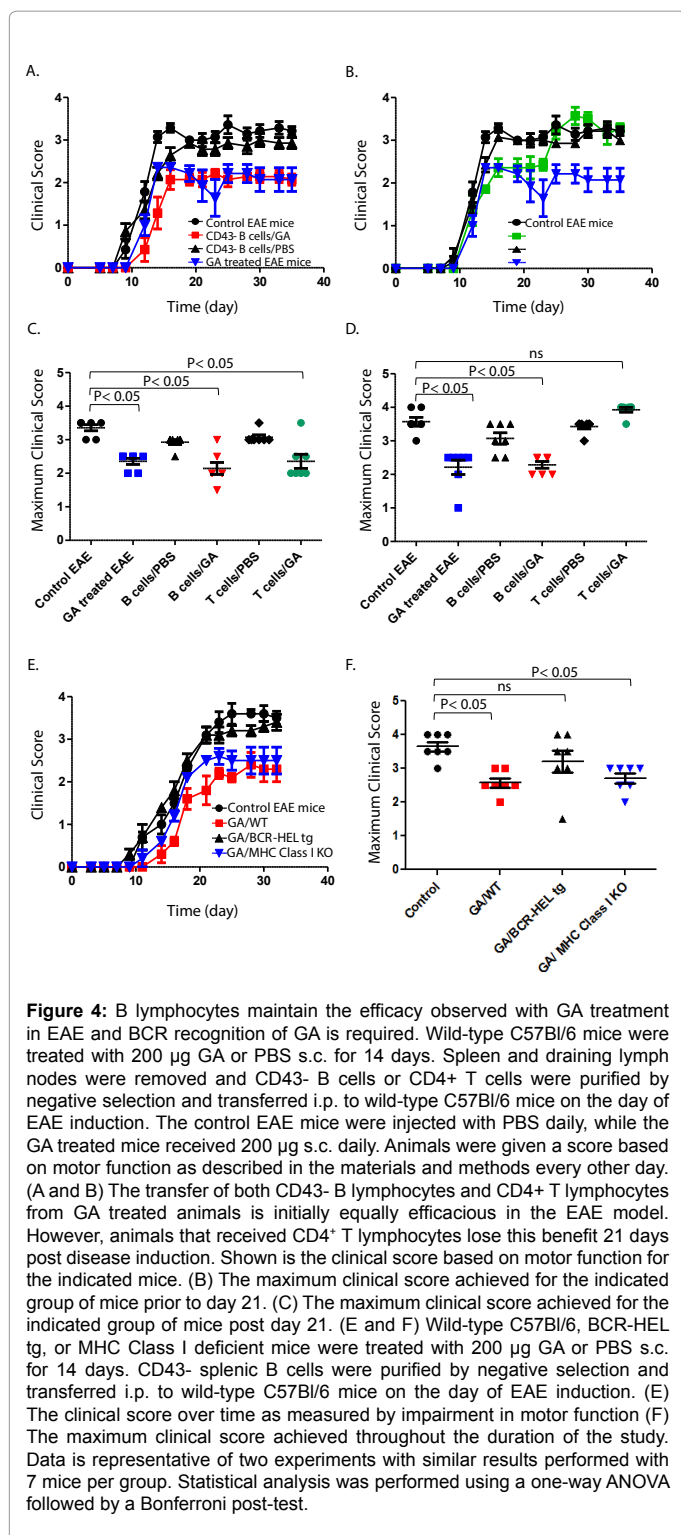
at one-hour post incubation, at which point intracellular GA increased and extracellular GA decreased. At 18 hours post incubation, an increase in extracellular GA was detected suggesting that the drug had been internalized and recycled back to the surface of B lymphocytes (Figure 2A bottom panel). To determine if GA bound to the BCR externally, and was recycled back to the surface of B lymphocytes through MHC Class II, the same binding study was performed with B lymphocytes from BCR-HEL tg mice and MHC Class II deficient mice. As expected, there was a significant reduction in the detection of extracellular GA on B lymphocytes from BCR-HEL tg mice, while there was a noticeable but insignificant increase in extracellular binding to MHC Class II deficient B lymphocytes at baseline (Figure 2B top panel). Extracellular GA on MHC Class II deficient B lymphocytes was internalized as shown by the decrease in extracellular GA (Figure 2B top panel) and an increase in intracellular GA (Figure 2B bottom panel), however it failed to be

recycled back to the surface of the cells, suggesting that the increase in extracellular GA observed 18 hours post GA incubation was GA in the context of MHC Class II (Figure 2B bottom panel). The ability of GA to bind to the BCR, undergo processing and be presented by MHC Class II on B lymphocytes suggests that B lymphocytes serve as a professional APC source for GA. To evaluate if B lymphocytes are competent APCs for GA, isolated splenic B lymphocytes were incubated with GA for 24 hours, washed and co-cultured with purified splenic CD4⁺ T cells. Ovalbumin was used as a positive control for the assay. B lymphocytes presenting GA induced CD4⁺ T cell activation as shown by IL-2 production in WT, but not in Class II deficient animals (Figure 2C). However, CD4⁺ T cell activation was not completely abrogated with BCR-HEL tg B lymphocytes suggesting that there are alternative modes of GA internalization in addition to the BCR, or alternatively, since the BCR in these mice is a transgene, it is possible that GA binds to a very small percentage of BCRs that are not HEL specific.

GA induces signaling on B cells, upregulates early activation markers, but fails to induce expression of late activation markers

To further explore the direct effects of GA on B lymphocytes, the ability of GA to upregulate activation markers CD69, CD86 and MHC Class II, induce intracellular Ca²⁺ release and phosphorylation of tyrosine's (pTyr), all indicative of a fully activated B cell was evaluated. Initiating the cascade of B cell activation includes the phosphorylation of tyrosine residues (pTyr) resulting in intracellular Ca²⁺ release. Initially, western blot analysis was used to detect changes in the banding pattern or induction of pTyr, however since only 5-17% of B lymphocytes bind GA, density analysis of the pTyr bands failed to produce robust results. Therefore, flow cytometric analysis of intracellular staining of pTyr on gated GA⁺ and GA⁻ splenic B lymphocytes was utilized. The disadvantage to this approach is that it does not distinguish the banding pattern of pTyr induced by GA, which may be different than anti-IgM induced BCR cross-linking. Increased pTyr induced by anti-IgM was used as a positive control for the assay (Figure 3A left panel). GA induced the upregulation of pTyr at 1 minute and 5 minutes post GA incubation on GA⁺ gated cells, but not on GA⁻ gated cells (Figure 3A right panel). The magnitude of this response was diminished compared to anti-IgM stimulation, suggesting that BCR specific effects of GA result in reduced signal strength as compared to cross-linking of the BCR with a high affinity monoclonal antibody (Figure 3A left panel). Consistent with B cell activation, an increase in intracellular Ca²⁺ was also observed in GA⁺ but not GA⁻ B lymphocytes (data not shown).

Activated B lymphocytes reach peak induced expression of CD69 (an early activation marker) 24 hours post activation. In preparation for CD4⁺ T cell activation, MHC Class II and CD86 (later activation markers) expression on B lymphocytes peaks 48 hours post activation. Anti-IgM stimulation alone is capable of upregulating the early activation marker CD69, and the later markers CD86 and MHC Class II. However, this induced expression is shorter in duration and decreased in magnitude when co-stimulation, such as CD40 ligation, is absent. The goal of this experiment was to determine the effects of GA alone on expression of these markers; therefore GA stimulation was compared directly to anti-IgM stimulation in the absence of co-stimulatory signaling. GA induced the expression of the early activation marker CD69, but failed to upregulate surface expression of MHC Class II and CD86 (Figures 3B and 3C). This could be due to the rapid internalization of GA, thus limiting the signal duration, or alternatively GA has reduced signal strength as compared to the anti-IgM antibody. Additionally, pre-incubation with GA inhibited the ability of anti-IgM



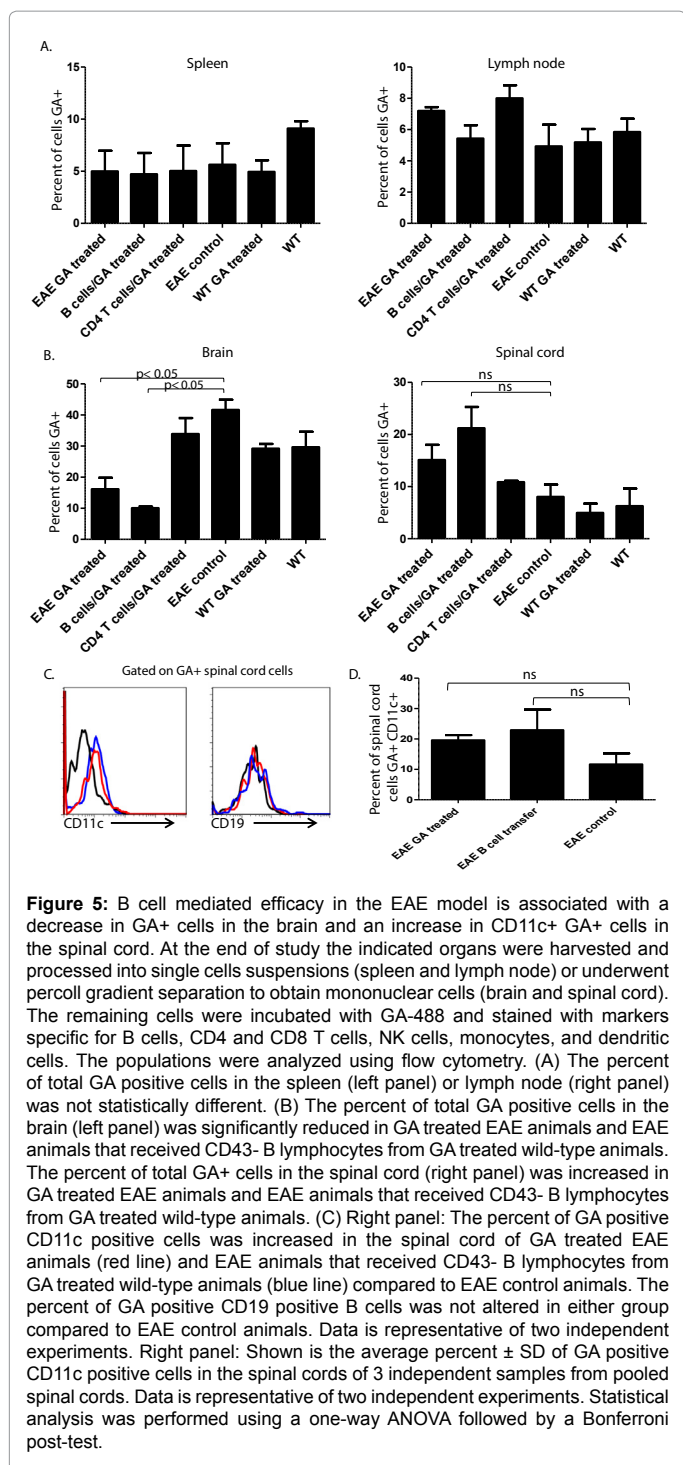
B lymphocytes maintain the efficacy observed with GA treatment in EAE and BCR recognition of GA is required

The protective effects of GA in EAE have been reported to occur through regulatory CD4⁺ T cells, monocytes, B cells, and other cells. The goal of this experiment was to determine the potency of the few cells mediating GA's efficacy by transferring the entire population of the immune cell of interest into MOG 35-55 immunized EAE mice. Initially, two separate populations of B cells were transferred using pan B cells or CD43- B cells. B cells isolated using the pan B cell kit dramatically enhanced disease severity and the transfer of these cells from GA treated mice did not reduce disease severity suggesting that highly pathogenic B cells reside within this B cell population and are unaffected by GA treatment (supplementary Figure 2). To evaluate the potency of lymphocytes capable of mediating the protective effect of GA treatment, wild-type mice were treated for two-weeks with 200 µg GA s.c., and total draining lymph node CD4⁺ T cells or total splenic CD43- B cells were isolated by negative selection from GA or PBS treated animals. Purified CD4⁺ T cells or CD43- B cells (5×10⁶ cells of each population) were transferred i.p. into EAE recipients on the day of MOG 35-55 immunization (Figures 4A and 4B). Sufficient quantities of blood monocytes were unable to be obtained from peripheral blood to perform a head-to-head analysis of efficacy. Other groups using monocytes expand these cells *in vitro* prior to transfer, which we chose not to do for our global T cell and B cell transfers, as this would hinder our ability to properly compare groups. Given the data generated on GA effects on B lymphocytes, it is possible that monocytes may also contribute to efficacy by a similar mechanism. Prior to day 21 post EAE induction, both CD43- B and CD4⁺ T lymphocytes transferred from GA treated mice significantly reduced the maximum clinical score by 36% and 29% respectively, comparable to the GA treated group with a 29% reduction (Figure 4C). However the CD4⁺ T cell mediated decrease in the maximum clinical score was abolished 21 days post EAE induction (Figure 4D). The maximum clinical score obtained post day 21 of GA treated EAE animals was reduced by 38%, while recipients that received B lymphocytes transferred from GA treated mice was reduced by 36% compared to EAE control animals, both of which were statistically significant (Figure 4B right panel). The average day of disease onset was significantly later in recipient mice receiving B lymphocytes from GA treated animals compared to controls (supplementary Figure 1). As most of the GA positivity observed in the GA^{hi} population was a reflection of an interaction between GA and the BCR (Figure 1), we hypothesized that this interaction is required for the observed efficacy. To determine if BCR recognition of GA is required for efficacy, CD43- B cells were transferred from wild-type, MHC Class I KO or BCR-HEL tg mice that had been treated with 200 µg s.c. of GA for two weeks to EAE recipients on the day of disease induction (Figure 4E). EAE recipients who received B cells from BCR-HEL tg animals had a non-significant reduction in the maximum clinical score (10%), compared to recipients who received B cells from GA treated wild-type donors (30%) or GA treated MHC Class I deficient donors (26%) (Figure 4F). Although a small proportion of GA^{hi} B lymphocytes represents GA binding to MHC Class I, B cells from GA treated MHC Class I deficient donors transferred to EAE recipients did not significantly enhance disease severity (Figure 4F). This data suggests that the major mechanism of GA induced B cell mediated efficacy occurs through a specific interaction between GA and the BCR.

B cell mediated efficacy in the EAE model is associated with a decrease in GA+ cells in the brain

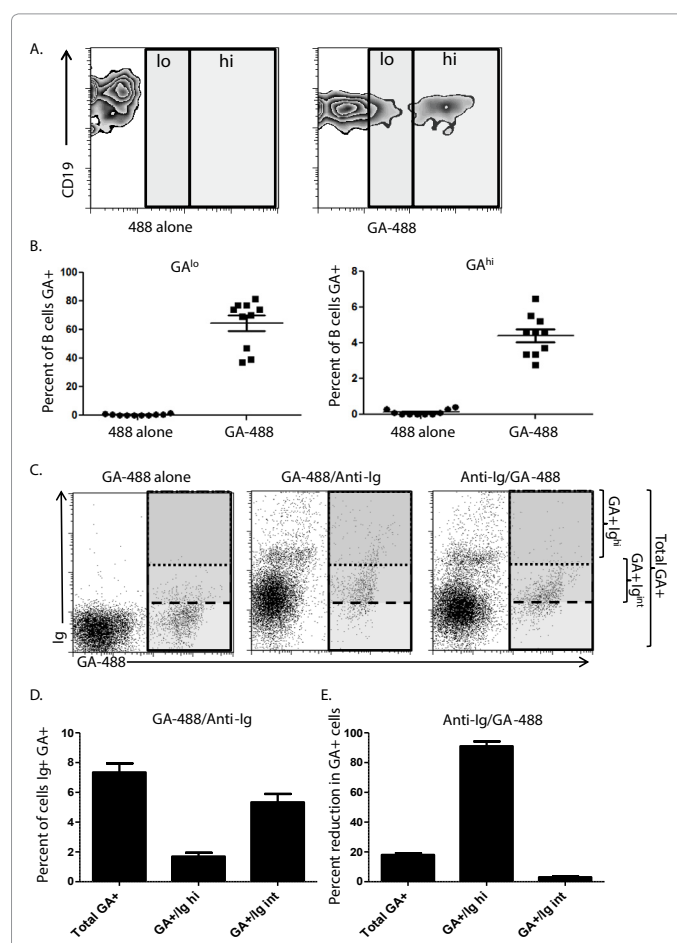
As the transfer of B lymphocytes from GA treated donors into

to induce the upregulation of CD86 and MHC Class II on GA+ but not GA- B lymphocytes, suggesting that GA blocks the binding of anti-IgM (Figure 3C). Although GA does not induce the upregulation of molecules synonymous with APC function, this did not hinder the ability of B cells to activate GA specific CD4⁺ T lymphocytes, suggesting that sufficient quantities of these molecules were present (Figure 2C).



EAE recipients was equally efficacious to GA treated EAE animals, we hypothesized that there may be an expansion or migration of GA specific B lymphocytes in the brain or spinal cord of these animals. The observed efficacy in the animals receiving B lymphocytes from GA treated WT mice, as well as GA treated EAE mice was accompanied by a noticeable but non-statistically significant increase in total B lymphocytes in the spinal cord, and a decrease in the brain (supplementary Table 1). Total inflammatory cells were not altered in the spleen or lymph nodes in all groups, and the percentage of

GA+ cells in these peripheral immune organs was similar (Figure 5A top panels and data not shown). Consistent with a reduction of B lymphocytes in the brain, the percentage of GA+ cells was significantly reduced in GA treated animals and EAE recipients who received B cells from GA treated donors (Figure 5B bottom left panel). In the spinal cord, the increase in total B lymphocytes in both of these groups was accompanied by a notable but statistically insignificant increase in GA+ cells, suggesting that GA+ B lymphocytes migrated to or expanded in the spinal cord (Figure 5A bottom right panel). To determine if GA+ B lymphocytes were increased in the spinal cord of these animals, mononuclear cells obtained by percoll gradient homogenized spinal cord were pre-incubated with GA-488 and stained with antibodies identifying various immune cell populations. Despite an increase in spinal cord B lymphocytes correlating with efficacy, the percentage of GA+B lymphocytes in the spinal cord was not altered (Figure 5C right



GA+ cells in these peripheral immune organs was similar (Figure 5A top panels and data not shown). Consistent with a reduction of B lymphocytes in the brain, the percentage of GA+ cells was significantly reduced in GA treated animals and EAE recipients who received B cells from GA treated donors (Figure 5B bottom left panel). In the spinal cord, the increase in total B lymphocytes in both of these groups was accompanied by a notable but statistically insignificant increase in GA+ cells, suggesting that GA+ B lymphocytes migrated to or expanded in the spinal cord (Figure 5A bottom right panel). To determine if GA+ B lymphocytes were increased in the spinal cord of these animals, mononuclear cells obtained by percoll gradient homogenized spinal cord were pre-incubated with GA-488 and stained with antibodies identifying various immune cell populations. Despite an increase in spinal cord B lymphocytes correlating with efficacy, the percentage of GA+B lymphocytes in the spinal cord was not altered (Figure 5C right

panel). Interestingly, the observed increase in GA⁺ spinal cord cells was due to an increase in GA⁺ CD11c⁺ cells (Figures 5C left panel and 5D). This increase accounted for the increase in total GA⁺ cells in the spinal cord, although this finding, similar to the increase in GA⁺ cells, was not statistically significant. This data suggests that GA induced B lymphocyte mediated efficacy observed in the EAE model may initiate with these cells, however other cell types may be required to maintain the efficacy.

GA binds to the BCR on human B cells from MS patients

To determine if our murine findings translate to humans, GA binding was evaluated on human PBMCs from 10 MS patients. Consistent with our murine results, an average of approximately 5% of human B lymphocytes bound high levels of GA termed GA^{hi} (Figures 6A and 6B). Also observed was the shift in the negative population, termed GA^{lo} (Figures 6A and 6B). Interestingly, there appeared to be two separate groups of patients with regards to GA^{lo} B lymphocytes. One cohort comprised of 3 patients had 37.1%, 38.9% and 46.9% of their B lymphocytes stain GA^{lo}, while 7 patients all showed GA^{lo} positivity ranging from 70% to 81% of their B lymphocytes (Figure 6B left panel). As discussed briefly in the murine binding studies, it remains unclear if this GA^{lo} population is an assay artifact or if this result is indicative of alternative mechanisms by which B lymphocytes obtain GA. Similar to the murine data, GA also bound to a small percentage of human CD4⁺ T cells, monocytes, few CD8⁺ T cells, and virtually no natural killer cells (data not shown). To determine if the GA^{hi} population of B cells represented GA bound to membrane bound BCR, a competition assay was performed using anti-Ig. The caveat to this study is that the ability of the antibody to block GA binding is unknown, as this antibody binds to all isotypes of Ig in a location that may not compete with GA, or induce steric hindrance. GA-488 or anti-Ig was pre-incubated with purified human B lymphocytes from 6 MS patients for 30 minutes, followed by several washes, and then the reciprocal was incubated with the cells for 20 minutes. GA positivity was quantified by flow cytometric acquisition and analysis (Figure 6C). Anti-Ig pre-incubation successfully prevented the binding of GA on GA⁺ Ig^{hi} B lymphocytes (Figures 6C and 6D) Pre-incubation with GA-488 did not prevent anti-Ig from binding (Figures 6C and 6D). This data suggests that GA⁺ Ig^{hi} positive cells are a representation of GA binding to the BCR on human B cells. The remaining GA^{hi} positive B lymphocytes may also represent GA binding to membrane Ig, however it is difficult to perform an assay on human cells to make that conclusion.

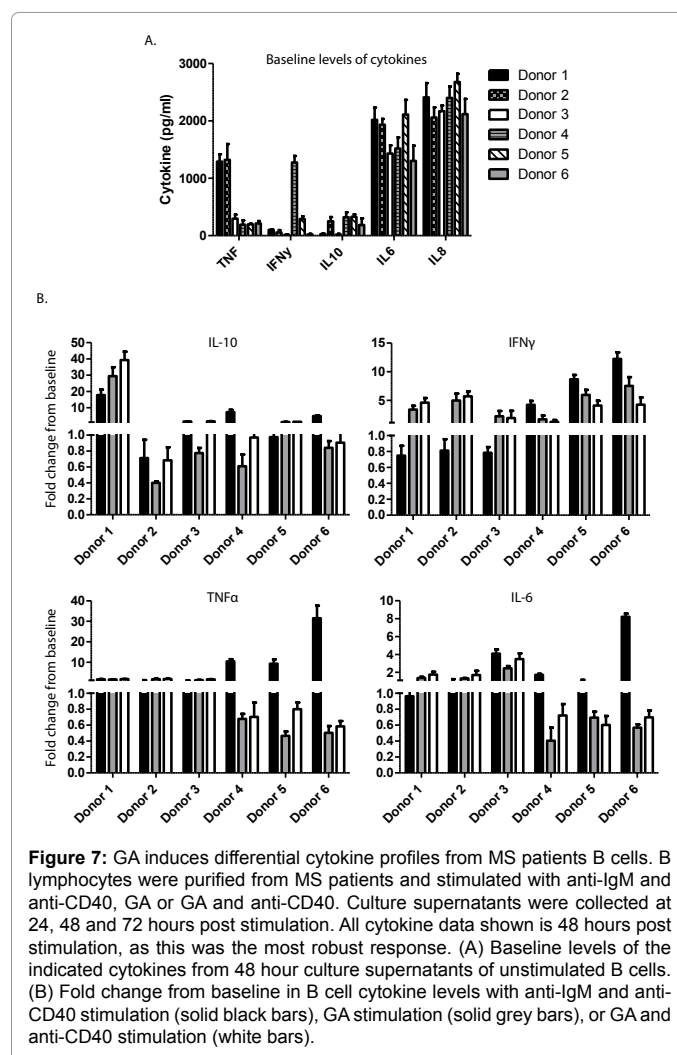
GA induces differential cytokine profiles from MS patients B cells

Although GA loaded murine B lymphocytes presented GA to CD4⁺ T cells resulting in IL-2 production, the transfer of CD4⁺ T cells from GA treated donors to EAE recipients failed to maintain efficacy post day 21. This suggests that T cells alone are insufficient to maintain efficacy and other mechanisms such as cytokine production from B lymphocytes may be required. To determine the cytokine profiles of GA stimulated B lymphocytes, purified B cells from treatment naïve MS patients were incubated with GA in the presence or absence of co-stimulation and soluble factors were measured in culture supernatants. A panel of cytokines including IL-1 β , IL-12-p70, IL-2, IL-4, IFN γ , TNF α , GM-CSF, IL-10, IL-6, and IL-8 were quantified 24, 48, and 72 hours post the indicated treatment. The best time-point for each cytokine was chosen based on the ability of the positive control, anti-IgM and anti-CD40 stimulation, to upregulate the indicated cytokine. The quantity of B lymphocytes obtained post negative selection was inadequate to perform an anti-IgM alone stimulation. As expected, the

quantities of IL-2, IL-4, IL-1 β , IL-12-p70 and GM-CSF were very low on the standard curve or below the limit of detection and were unaltered in the conditions tested (data not shown). The quantity of IL-8 appeared to be high in all donors tested, however these levels were not changed in any of the conditions tested (Figure 7A and data not shown). Figure 7A shows the baseline levels for all of the cytokines in which a change was observed, or in the case of IL-8, high basal levels were measured. All donors tested appeared to have high levels of baseline IL-6, while the levels of the other cytokines were within the standard range for unstimulated cells (Figure 7A). There was no clear pattern observed with IL-10 production from B lymphocytes with any of the donors, while all donors upregulated IFN γ in response to GA treatment (Figure 7B top panels). Interestingly, in three of the six donors, TNF α and IL-6 were reduced below baseline levels at all time-points tested with GA and GA + anti-CD40 treatment, while these cytokines were increased with anti-IgM and anti-CD40 treatment (Figure 7B bottom panels). This data suggests that the effects of GA induced cytokine production on B lymphocytes in humans is donor specific, and may guide future studies evaluating if this dichotomy could serve as a biomarker for optimal Copaxone responders.

Discussion

The novel findings in this study showing that GA induces differential



cytokine production in treatment naïve B cells from MS patients, is an antigen recognized by human and murine B cell receptors and this interaction is required for efficacy in mice may support treatment decisions for optimal Copaxone responders, and/or lay the ground-work required for synergistic combination therapies in MS. Recognition of an antigen through the BCR results in several non-exclusive outcomes including presentation to T cells, cytokine production, and upregulation of molecules involved in communication with other cells. It is likely that the efficacy observed prior to day 21 in EAE recipients who received CD4⁺ T cells from GA treated donors is a reflection of these cells responding to GA in the context of MHC molecules from B cells. Post day 21, only animals who received B cells and not T cells from GA treated donors maintained efficacy equivalent to GA treated EAE mice. This suggests that B cells are able to initiate and maintain efficacy, while T cells alone are insufficient. If this is true, then it is unlikely that the only mechanism of action of GA on B lymphocytes driving efficacy is antigen presentation and may involve cytokine production and/or other mechanisms involved in cellular communication.

The evidence to support B cell dependent cytokine production as an additional mechanism of efficacy is that in B cells from treatment naïve MS patients, GA in the presence or absence of anti-CD40 costimulation altered cytokine profiles. It is likely that GA modulation of cytokines in humans also occurs through the BCR, as pre-incubation with anti-Ig reduced the proportion of GA^{hi} positive B cells. GA reduced the pro-inflammatory cytokines TNF α and IL-6 below baseline levels in 50% of the patients tested. IL-6 secreted from B lymphocytes has been implicated in EAE pathogenesis and CNS pathology in mice [60-63]. In MS, a role for IL-6 in pathogenesis has been reported, however the source and mechanism of action remains unclear [64-71]. Therefore, the observed reduction in patients with GA treatment may be a contributing factor to its efficacy. The role of TNF α in CNS disease is paradoxical, with reported protective and pathologic roles. In rodents, TNF α is mostly damaging to the CNS with deleterious effects reported on astrocytes, oligodendrocytes, myelin, and axons [72-89]. In MS patients, neutralization of TNF α with a specific monoclonal antibody or a soluble TNF α receptor exacerbated clinical symptoms, suggesting in humans this cytokine is likely protective [90,91]. Although GA reduced baseline levels of TNF α in three of the six B cells from MS patients, there are no reports of Copaxone enhancing disease severity, suggesting that this reduction is not sufficient to mimic the deleterious effects observed with complete neutralization of TNF α . Alternatively, TNF α may not be reduced in patients taking Copaxone. Studies performed quantifying cytokines levels from MS patients on GA treatment either compared the results to natalizumab treated individuals, or the focus was on T cell cytokines [92,93]. Future studies may include determining if pro-inflammatory cytokines from B cells are reduced in MS patients taking Copaxone compared to untreated individuals, and if this correlates with efficacy.

The evidence to support communication with non-T cells as a mechanism of B cell mediated efficacy originates with the EAE data. Although this data showing an increase in GA⁺ lymphocytes in the spinal cord from GA treated EAE mice, and EAE recipient mice receiving B lymphocytes from GA treated donors was not statistically significant, the increase in GA⁺ CD11c⁺ cells from both groups was consistent. CD11c is expressed on dendritic cells, monocytes, macrophages, neutrophils and some B cells. The percoll gradient removed neutrophils and the CD11c positive population was CD19 and F480 negative, thus excluding CD11c⁺ B cells, macrophages and microglia. Therefore it is likely that these cells are either dendritic cells or monocytes. One reported mechanism of GA induced efficacy is

through anti-inflammatory type II monocytes, however these cells are CD11c negative excluding this population [25,39]. CD11c⁺ dendritic cells have been implicated in GA mediated efficacy *in vivo* promoting Th2 development and increasing IL-10 production [94-98]. Therefore, it is likely that the observed increase in CD11c⁺GA⁺ cells in the spinal cord of GA treated mice or recipient EAE mice who received B lymphocytes from GA treated donors represents an anti-inflammatory dendritic cell population. B cells are capable of directing innate immune cells mostly through cytokines; however other mechanisms are possible [99-103]. Given that a B cell receptor specific for GA is required for B cell driven efficacy, and the increase in CD11c⁺ GA⁺ cells in the spinal cord, it is conceivable that B lymphocytes transferred from GA treated mice results in efficacy through a BCR dependent anti-inflammatory cytokine interaction with dendritic cells effectively reducing disease severity.

In conclusion, the data reported in this manuscript has potentially two major clinical applications. The first is determining if GA induced B cell cytokine profiles or an increase in GA⁺CD11c⁺ cells post-treatment, predicts optimal Copaxone responders. Future studies may include confirming the GA induced dichotomous cytokine findings in a large cohort of patients, and determining if this effect correlates with efficacy. The dual GA binding populations is quite interesting and could possibly be used to stratify high vs. low responders, as there was a clear-cut difference in the percentage of GA^{lo} B lymphocytes in MS patients. As stated earlier, although the significance of the increased CD11c⁺ GA⁺ cells in the spinal cord of mice benefiting from GA remains unknown, this finding may also have clinical implications in predicting optimal responders or support highly effective combination therapeutics. The second is evaluating if the data generated can guide highly efficacious synergistic therapeutic combinations for MS patients. Sub-optimal responders to Copaxone may greatly benefit from safe synergistic combination therapies such as Laquinimod. Currently in clinical trials for MS, Laquinimod has been reported to result in similar effects of GA including altering dendritic cells, inducing type II monocytes, increasing T and B regulatory cells, decreasing antigen presentation, and direct neuroprotective effects [104-117]. The novelty of this study is that a direct functional effect of GA on B lymphocytes has not been previously reported. This data provides the ground-work necessary to predict optimal Copaxone responders and combination therapy development potentially resulting in dramatically enhanced efficacy while maintaining a favorable safety profile.

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