High dose antigen treatment with a peptide epitope of myelin basic protein modulates T cells in multiple sclerosis patients

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One of the auto-antigens aberrantly targeted in Multiple sclerosis is myelin basic protein (MBP). In this study, chronic progressive multiple sclerosis (CPMS) patients receiving the experimental drug MBP8298, on a compassionate care trial, were examined before and after high dose peptide treatment for their circulating regulatory T-cell numbers and their responses to the common mitogens, phytohemagglutinin and poke-weed mitogen. Peripheral blood mononuclear cells (PBMCs) isolated from these patients before treatment displayed anergy upon stimulation with phytohemagglutinin; measured through reduced proliferation, IFN-γ and IL-17A secretion in an in vitro cell culture system. 6 Weeks and 6 months after treatment their PBMCs displayed a reversal of anergy with phytohemagglutinin stimulation. There was also a marked increase in their CD4+CD25+FoxP3+ T-cells regulatory T-cells. These results suggest that high dose MBP8298 treatment has a profound effect on the circulating T-cells of CPMS patients, capable of reversing peripheral anergy and establishing T regulation.

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1. Introduction

Multiple sclerosis (MS) is a complicated autoimmune disease affecting the central nervous system of patients often leading to neurological disability. In a 2005 study it was estimated that over 240 of every 100 000 Canadians are afflicted by MS [1]. This leaves Canada with some of the highest incidences of MS in the world. Progressive forms of MS are especially debilitating because there are no signs of remittance during this course of MS. The patients suffer a constant increase in their disability over time (MS Society of Canada). MRI scans of the brain and central nervous system (CNS) characterize areas of active neural degradation in 'plaques'. These plaques are sites of active inflammation harboring infiltrating immune cells, especially T cells [2].

The etiology of disease incidence and progression of MS is not clear yet, and genetic, immunologic, environmental and infectious factors have been suggested. Autoimmune responses have been suspected as a major cause of MS. The presence of CD4+ and CD8+ T cells in neuronal plaques and the even distribution of MHC class II positive microglia cells implicate an immune involvement in the disease [3]. Myelin basic protein (MBP) reactive T cells have been found in the blood of both MS patients and healthy volunteers however, it is suggested that the inflammatory phenotype of those T cells could be greater in diseased individuals [4]. The importance of T cell autoreactivity to MBP in MS disease is supported by mouse models of MS where the administration of MBP with inflammatory adjuvants, such as complete Freund's adjuvant into mice initiates an experimental autoimmune encephalomyelitis (EAE) that largely resembles MS [5]. MBP immunodominant regions have also been characterized through epitope mapping of B and T cells from MS patients [6].

Currently the treatments offered to MS patients have focused on establishing an immunosuppressive environment or altering the type of T cell response in their CNS. IFN-β1b was one of the first treatments utilized against MS [7]. IFN-β functions as an immunosuppressive cytokine. Since the etiology of MS is still unknown it seemed logical to suppress all immune functions to limit neuroinflammation. This belief has led to a number of off label uses of immunosuppressive drugs; these include cyclophosphamide, azathioprine, methotrexate [8]. Immunosuppressive agents however, are known for their numerous side effects including inhibition of the routine clearance of common pathogenic bacteria and in severe cases, hepatotoxicity. Glatiramer acetate (GA), a synthetic peptide designed to most closely resemble encephalitogenic properties of MBP, has been used to treat MS. GA has been shown to alter the T cell response from a Th1 to Th2 while also having immunomodulatory properties. Subcutaneous administration of GA has been approved for treatment of relapsing-remitting MS, based on favorable clinical and imaging data in clinical trials [9]. These results have encouraged the search to develop selective immune therapies
against MS which largely target modes of immune activation [10]. These include monoclonal antibodies against B-cells and common immune synapse associated adhesion markers [8]. Cost and limited success remain an issue in wide-spread acceptance of monoclonal antibody therapies.

One treatment that has been considered to re-establish a tolerogenic environment within the CNS utilizes the phenomenon of high dose antigen tolerance [11]. The approach uses a peptide consisting of the T and B-cell immunodominant epitope found in HLA DR2 MS patients [6]. A very successful phase 2 clinical trial was conducted that showed a strong clinical benefit in treatment for MS, measured by the expanded disability status scale (EDSS) [6]. The peptide, located within MBP, consists of the amino acid sequence between 82 and 98. It was found that there was no correlation between EDSS scores and MBP specific antibody titres [6].

A link between the number of circulating regulatory T cells (Tregs, CD3+CD4+CD25+hi) and MS disease has been disputed among researchers for some time. In a recent review on Tregs in MS, it was discussed that some investigators report a lower number of circulating Tregs while others do not [12]. It is generally understood that Tregs from MS patients appear to have a defect in their ability to suppress an immunological response [13]. Studies performed on mice show the important role that Tregs play in the resolution of an EAE inflammation episode [14]. Their numbers are also seen to be elevated in cancer patients [15], which are shown to be responsible for the tolerogenic environment that exists within tumors and prevents successful immune clearance of tumors.

We, therefore, hypothesized that upon high dose administration of MBP8298 there will be an increase in the number of circulating Tregs or a reversal in their unresponsiveness. Our results demonstrated an increase in the number of Tregs in patients' peripheral blood mononuclear cells 6-weeks and 6-months post treatment with high dose of MBP peptide. We also unexpectedly observed evidence of peripheral T-cell anergy in untreated patients and the ability of MBP8298 treatment to reverse the T cell anergy state.

2. Methods

2.1. Patients

10 chronic progressive MS patients selected to receive MBP8298 based on compassionate care admission were selected for this study by Dr. Kenneth Warren (University of Alberta Hospital). Patients were given 500 mg of MBP8298 (obtained from BioMS Medical Corp., Edmonton, Canada) intravenously every 6 months until the end of their prescribed study. Blood was drawn before their first treatment with MBP8298, 6 week follow-up visit and 6 months after treatment prior to their next MBP8298 administration. The use of patients’ blood samples was approved by the Human Research Ethics Board at the University of Alberta.

2.2. Cell culture

PBMCs from whole blood were isolated using Ficoll-paque (R&D Systems, USA) density gradient separation. PBMCs were then plated at 2×10⁶ cells/well in a standard 96 well tissue culture plate. Cells were treated in triplicate with 1 μg/mL of phytohemagglutinin (PHA), poke weed mitogen (PWM) (Sigma, USA) in AIM-V media (Invitrogen, USA). They were incubated at 37 °C and 5% CO₂ for 3–4 days before supernatant collection. Supernatant was collected by pooling from the wells that were given the same mitogen and samples were frozen at −80 °C until required. Cells were then immediately pulsed with 0.5 μCi/well of [³H]thymidine (Amersham Pharmacia, Canada) overnight and then harvested onto micro-cellulose membranes to determine thymidine uptake into proliferating cells.

2.3. Cytokine ELISAs

IL-17A, TGF-β and IFN-γ were measured using ELISA kits following manufacturer’s procedures (Bioscience, USA and Biosource, Canada). Culture supernatants were diluted 1/10 so that absorbance would fit on the standard curve produced by the standards run on each plate.

2.4. Flow cytometry

PBMCs were stained with antibodies Q4120 (anti-CD4) labeled with FITC (Sigma, USA), M-A251 (anti-CD25) labeled with PE (BD Pharmingen, USA) and 236A/E7 (FoxP3) labeled with APC (ebioscience, USA) along with corresponding isotype controls. Cells were stained in 5 ml flow cytometry tubes (BD Falcon, USA) where they were blocked before staining with 5% human AB serum in staining buffer. Staining was performed on ice for 30 min in the dark. Cells were then washed and fixed in 2% (w/v) parafomaldehyde (Sigma, USA) before permeabilization with 0.3% (w/v) saponin buffer (Sigma, USA). Intracellular staining for FoxP3 was carried out for another 30 min in the dark on ice. Cells were fixed and analyzed within 24 h on a FACSCanto (BD, USA).

2.5. Healthy controls

Healthy control blood was obtained from normal donors. Nine different donors were analyzed together to generate the healthy control group. 2 × 10⁶ PBMCs were plated in standard tissue culture treated 24 well plates in 1 mL of AIM-V media. Culture supernatant was harvested on day 3 and cytokines were tested. To compare cytokine concentrations with the MS test groups, concentrations were divided by half to make comparable concentrations.

Three healthy donors were tested for their CD4 and CD25 expression using the same flow cytometry staining protocol used on MS patient samples.

2.6. Statistics

Patient data was pooled and tested using a standard one-way ANOVA followed by a tukey post hoc test to determine individual relationships between groups. SPSS (IBM, USA) statistical software was used to make the calculations.

3. Results

3.1. Increase in the percentage of CD4+CD25hi cells in PBMCs of patients after treatment with MBP8298

Whole PBMCs obtained from patients before treatment, and 6-weeks and 6-months post-treatment were stained for CD4/CD25 expression (Fig. 1). Overall the CD25 hi T cells population was significantly increased in the CD4 T cells 6-weeks post-treatment, and an increasing trend was maintained after 6-months. These results suggest an overall activation of CD4 T cells after treatment with high dose of MBP8298. However, since CD25 cannot be used as a sole marker for Tregs, because activated T cells also express CD25, we used hi-CD25 expression as a marker for Tregs as has been suggested by Baecher-Allen et al. [16]. We compared the frequency and percentage of circulating CD4+CD25hi cells before and after treatment. We found that the CD25 hi population became more abundant in both time periods post treatment compared to before treatment with MBP8298 (Fig. 1a). The increase post treatment
was significant when data from all 10 patients enrolled in this study were pooled together (Fig. 1b). Interestingly, the population of circulating CD4⁺CD25⁺ cells in healthy patients did not differ from pre-treatment frequencies in MS patients. We also found that the average expression levels of CD25/IL2R on CD4⁺ cells increased after treatment, approaching the levels seen in healthy individuals (Fig. 1c).

Human Tregs were first characterized as CD4⁺CD25⁺ T cells by several groups in 2001 [17,18]. Transcription factor Forkhead box P3 (FoxP3) was first described as a master control gene for mouse Treg cell development and function [19]. Subsequently, FoxP3 was shown to be a marker for human Tregs as well [20]. However, in later studies, it was shown that both CD25 and FoxP3 expression could be induced in human naïve CD4⁺ T cells through T cell activation blurring the identification of FoxP3⁺ cells as pure Treg cells in humans. The expression of FoxP3 by both activated and regulatory CD4⁺ T cells in humans could be one component of homeostatic programming initiated by these cells to exert negative feedback during the course of an immune response [21,22]. To examine whether the increased CD25⁺ cells belong to the regulatory T cell subsets, we measured the level of FoxP3 expressed intracellularly. We found that there was a significant increase in the percentage of cells expressing FoxP3 6-months post treatment compared to pretreatment or 6-weeks post treatment groups (Fig. 2). The delay in the upregulation of FoxP3 could be due to the time it took the body to wind down autoimmune responses and re-establish a regulatory environment.

### 3.2. Treatment with MBP8298 reverses T cell hyporesponsiveness

Circulating PBMCs were cultured in the presence of pokeweed mitogen (PWM) and phytohemagluttinin (PHA) to determine the overall responsiveness of immune cells before and after high dose antigen treatment. The proliferation of these cells as well as the cytokines produced suggests that after treatment with MBP8298 they become more responsive to mitogen stimulation. This was especially true in the cultures treated with PHA compared to PWM. Proliferation of PBMCs against PHA is statistically lower in pretreatment cells compared to either of the post treatment time frames (Fig. 3). The delay in the upregulation of FoxP3 could be due to the time it took the body to wind down autoimmune responses and re-establish a regulatory environment.
MBP8298 suggests that the treatment has reversed the hyporesponsiveness that was originally present.

IL-17A, which is a cytokine initially found to be important in autoimmune disorders and only now being studied for its role as an effector cytokine, was found to be in lower levels pre-treatment as well (Fig. 4). However, substantial amounts of IL-17A were found in the culture supernatants of cells stimulated with PHA in after treatment samples. This further supports our belief that high dose antigen treatment is modulating the T cell responsiveness.

TGF-β, a regulatory cytokine, was interesting in that, in the cells cultured with PHA in the pretreatment samples, relatively high levels of TGF-β were present (Fig. 4), whereas the amounts were almost undetectable in cells obtained after treatment.

4. Discussion

It has been recently reported that the phase 3 clinical trial with MBP8298 treatment for use on progressive patients has failed to reach statistical endpoints in EDSS scores when it was compared to placebo. This result was not anticipated after the successful phase 2 trial performed earlier [6]. The difference in the clinical results obtained between the phase 2 and 3 trials was suggested to be due to an improving EDSS score in the placebo treatment group in phase 3 trial [23]. Such setbacks are not uncommon in the field of clinical research developing treatments for MS [24]. Despite the clinical setbacks, the biological data from such clinical trials are still crucial in helping us understand biological responses to the treatment administered.

It is becoming widely understood that many autoimmune diseases, such as MS, could have their etiology based on an underlying immune dysfunction. There have been reports that in MS there exists a lack of immune suppression by CD25+ Treg cells and overall responsiveness by peripheral T-cells [13]. Our data clearly supports this observation by displaying a form of peripheral T-cell hyporesponsiveness in MS patients. There is also new evidence to suggest that an IL2R/CD25 polymorphism may exist that pre-disposes people to MS [25]. The polymorphisms found in the IL2R/CD25 gene locus could be responsible for CD25 having less affinity for IL2, but that remains to be tested.

The increase in CD25 expression after treatment with high dose self antigen would suggest that there is T cell regulation mediated through CD25. This population also appears to be increased in the
proportion of FoxP3+ cells which would indicate an increased num-
ber of circulating Treggs. Their appearance 6 months after initial

treatment may suggest that an active modulation of peripheral T
cells is taking place in the treated patients. An increase in CD25

on Tregs could aid in binding up free IL-2 through cytokine deple-
tion of active T cells in the central nervous system and circula-
tion [26]. The surge of CD25 could also help push the immune
system back into homeostasis or if the lower affinity IL-2 polymor-
phism did exist then an increase in CD25 on the cell surface could
potentially help combat that. It is also proposed that the increase in
CD25 could lead to better activation of Tregs through environmen-
tally available IL-2, stimulating their other suppressive activities
[27]. The reversal of peripheral T-cell hyporesponsiveness after treat-
ment with high dose antigen lends support to the initial idea that
this condition could prevent the body from re-establishing homeo-

stasis [28]. IFN-γ could be a principle cytokine that plays a role in
re-establishing homeostasis. It has long been seen as an effector
cytokine however, it may also act as an immune regulator/suppres-
sant in high concentrations [29]. This regulatory property of IFN-γ
is already being shown to be required in the treatment of a sys-
temic lupus in a mouse model [30]. If this mode of regulation was
lost on the circulating PBMCs before treatment, this would explain
any episode of uncontrolled inflammation. The response
observed in the healthy patients would further suggest a role for
IFN-γ to be a regulator against autoimmune disease. The return
of this regulation could prove important in future studies regarding
MS. This goes against the observations seen by Kinnman et al. who
found that the mitogenic reactivity of blood lymphocytes was not
different between healthy and MS patients [31]. This could be

because the groups they looked at did not involve MS patients with
advanced disease.

The induction of suppression is significantly influenced by cyto-
kines. The cytokines IFN-γ and IL-17A have also been correlated to
many autoimmune disorders including MS [32]. It is believed that
IL-17 producing T cells are naturally occurring and are somehow
stimulated to recognize auto-antigens. New evidence has chal-

lenged the role of IL-17 as an effector cytokine for autoimmune dis-

ease. The mice with induced EAE treated with an anti-IL-17
monoclonal antibody had a reduction in symptoms but not a total
abrogation of EAE lesions indicating that other cytokines must be
present to signal for inflammation [33]. Alternate theory now sug-
gests that IL-17 is a constituent of the normal course of inflamma-
tion and its expression occurs early on to strengthen an adaptive
immune response compared to other more studied cytokines such as
IFN-γ [34]. Our studies in contrast have shown that a reversal in
peripheral hyporesponsiveness restored the ability of circulating
PBMCs to produce IFN-γ and IL-17A. These cytokines may play
an important role in disease progression at the site of infection
but in the periphery they may play an equally important role in
returning homeostasis to the MS patient. It is curious that with
the increase in peripheral Treggs there is a decrease in TGF-β follow-

ing PHA stimulation. The source of TGF-β would need to be con-

firmed through further studies. However, the presence of TGF-β
after PHA stimulation from the pre-treatment group supports the
idea that it is aiding in maintaining peripheral T-cell hyporespon-
siveness. Its disappearance after treatment would indicate that T
cells producing IFN-γ and IL-17A have re-established homeostasis
in the periphery.

In this study we have shown that high dose treatment with a
known B cell recognizing auto-epitope of myelin basic protein is
capable of reversing the mitogenic hyporesponsiveness of PBMCs.
This reversal is also characterized by an increase in the number of
CD4+CD25hi cells detected in the circulation of these patients.
Whether these cells have the ability to suppress an immune re-

sponse remains to be seen. This research is the first to show the
reversal of immunological hyporesponsiveness in MS patients after
a high dose antigen treatment is administered. Our results suggest
that the regulation of T cells is an active process and require them
to be active and producing certain regulatory/effector cytokines.

If using other high dose antigens can return IFN-γ regulation and
increase the effectiveness of Treggs then we have a potential
new avenue to explore for treating autoimmune diseases with
deregulated IFN-γ. Diseases that lack IFN-γ regulation still need
to be defined because of the lack of focus afforded to the field by
our past discrimination of IFN-γ, believing it only acted as a stim-
ulating, effector cytokine.

A link regarding the number of circulating Treggs (CD3+CD4+ 
CD25hi) has been disputed among MS researchers for some time.
Many acknowledge that the circulating frequency of Tregs do not
differ between MS and healthy individuals, however, there does
appear to be a defect in the ability of these cells to suppress an
immunological response [13]. Our data would support the idea
that the defect could be overall T-cell hyporesponsiveness. It is
not disputed that Tregs can play a major role in re-establishing
and maintaining tolerance in models of autoimmunity and cancer
[14,15]. We now believe that circulating T cells, including Tregs,
in patients have simply become anergic and that high dose antigen
treatment can reverse their anergy and therefore increase their
CD25/IL2R expression and their function as Treggs.

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References


Laplaud, Frequency of circulating autoreactive T cells committed to myelin


MBP21-88 delayed disease progression in an HLA class II-defined cohort of
patients with progressive multiple sclerosis: results of a 24-month double-
blind placebo-controlled clinical trial and 5 years of follow-up treatment, Eur.

[7] D. Paolicelli, V. Direnzo, M. Trojano, Review of interferon beta-1b in the treat-
ment of early and relapsing multiple sclerosis, Biologics 3 (2009) 369–
376.


from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory


