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Glatiramer acetate-reactive T lymphocytes regulate oligodendrocyte progenitor cell number *in vitro*: role of IGF-2

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Abstract

Glatiramer acetate (GA) is an immunomodulator approved for therapy of relapsing-remitting multiple sclerosis (RRMS), but recent findings indicate that it may also have additional, neurotrophic effects. Here, we found that supernatants from human GA-reactive T lymphocytes potentiated oligodendrocyte numbers in rodent and human oligodendrocyte progenitor (OPC) cultures. Effects of Th2-polarized lines were stronger than Th1-polarized cells. Microarray and ELISA analyses revealed that neurotrophic factors induced in Th2- and Th1-polarized GA-reactive lines included IGF-2 and BMP-7 respectively, and functional studies confirmed IGF-2 as trophic for OPCs. Our results support the concept that GA therapy may result in supportive effects on oligodendrocytes in RRMS patients.

Keywords

multiple sclerosis; glatiramer acetate; T lymphocyte; oligodendrocyte progenitor; CNS repair

1. Introduction

Glatiramer acetate (GA) is a synthetic copolymer approved for therapy of the relapsing-remitting form of multiple sclerosis (RRMS), an autoimmune demyelinating disease of the CNS that is the most common nontraumatic cause of paralysis in young adults (Blanchette and Neuhaus, 2008; Wolinsky, 2006). Until recently, work on the mechanism of action of GA has focused primarily on its immunomodulatory effects on T lymphocytes and antigen presenting cells (APCs). GA has been shown to bind to major histocompatibility complex (MHC) molecules on the surface of APCs and prevent binding of myelin basic protein (MBP) and subsequent activation of T helper 1 (Th1) or Th17 encephalitogenic lymphocytes

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(Fridkis-Hareli et al., 1999; Fridkis-Hareli and Strominger, 1998; Gran et al., 2000). Interestingly, GA-reactive T cells are also known to be present in the circulation of most individuals, and are predominantly of a Th2 phenotype (Aharoni et al., 1997; Chen et al., 2002; Dhib-Jalbut et al., 2003; Neuhaus et al., 2000). Activation of these cells in the CNS leads to suppression of autoaggressive myelin-specific Th1 lymphocytes (Aharoni et al., 1998). GA-reactive Th2 lymphocytes are believed to act as key mediators of the beneficial effects of the drug in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, and have been shown to transfer protection to untreated animals (Arnon and Aharoni, 2004). GA treatment *in vivo* has also been shown to induce type-2 ('anti-inflammatory') myeloid cell differentiation (with consequent Th2 T cell differentiation) in both MS patients (Kim et al., 2004b) and in the context of EAE (Weber et al., 2007; Weber et al., 2004). The ability to induce CD4⁺ CD25⁺ regulatory T cells (Tregs) is also considered as one of the immune mediated therapeutic mechanisms of GA in EAE (Jee et al., 2007) as well as in a model of type 1 diabetes (Cui et al., 2009).

Recent findings have implied an additional, neurotrophic mechanism of action of GA in MS, suggesting an additional role for GA therapy beyond immunoregulation. In demyelinating models, GA treatment has been shown to result in elevated CNS levels of neurotrophic factors including brain derived neurotrophic factor (BDNF), neurotrophins-3 and -4 (NT3, NT4), and insulin-like growth factor-1 (IGF-1) (Aharoni et al., 2005; Skihar et al., 2009). Strikingly, human GA-reactive T lymphocytes have also been shown to express both BDNF and IGF-1 (Chen et al., 2003; Skihar et al., 2009; Ziemssen et al., 2002). While these factors have important effects on neurons, they are also significant regulators of oligodendrocyte lineage cells, which are the myelin-forming cells in the CNS and the target of immune attack in MS (Raine et al., 1997). Oligodendrocyte progenitor cells (OPCs) are the main source of new myelin in repairing lesions in MS (Bruck et al., 2003). IGF-1 potentiates the growth and differentiation of OPCs *in vitro* and increases brain growth and CNS myelination in transgenic mice *in vivo* (Carson et al., 1993; Hsieh et al., 2004). Similarly, mice lacking functional NT3, or its receptor trkC, are deficient in mature oligodendrocytes and OPCs (Kumar et al., 1998), and myelin formation is enhanced by NT3 in CNS co-cultures and in the injured CNS (McTigue et al., 1998; Yan and Wood, 2000). BDNF has also been shown to potentiate CNTF-induced oligodendrocyte survival *in vitro* (Barres et al., 1993). Although the capacity to produce neurotrophic factors is not unique to GA-reactive T lymphocytes (Stadelmann et al., 2002), these data suggest that factors produced by GA-reactive T lymphocytes may protect oligodendrocytes and/or enhance their differentiation and myelin production in the CNS of RRMS patients.

Here, we present results showing that factors produced by activated GA-reactive T lymphocytes regulate oligodendrocyte numbers in primary rodent and human OPC cultures. Our data suggest that multiple neurotrophic factors are involved in these effects, and implicate an additional member of the IGF family, IGF-2, as an important component of this response. These findings support the hypothesis that in addition to its well-recognized immunoregulatory properties, GA therapy may result in supportive or instructive effects on oligodendrocyte lineage cells in RRMS patients.

2. Materials and Methods

Tissue culture – lymphocytes

GA-reactive T cell lines were generated from peripheral blood of healthy adult controls as described (Kim et al., 2004a). PBMC were isolated by Ficoll-hypaque gradient centrifugation and plated in 96-well plates in medium containing 10 ng/ml IL-7 without antigen or with 20 µg/ml GA. After 7 days, medium was enriched with rhIL-2 (20 U/ml). On day 11, each well was evaluated for GA reactivity in a split-well assay. Positive wells were

defined as stimulation index (SI) >3 and proliferation > 3 SD above mean of 'no antigen' control wells. Confirmed GA-reactive wells were expanded as lines with rhIL-2 and restimulated regularly with GA and autologous irradiated PBMCs. Control lines were generated using polyclonal stimulation with OKT3 antibody in place of selection for GA reactivity. OKT3 binds to the epsilon subunit within the human CD3 complex (Van Wauwe et al., 1980). OKT3 hybridoma supernatant was used at a dilution such that OKT stimulation produced similar levels of T cell proliferation as GA stimulation, as assessed by CFSE dilution or tritiated thymidine incorporation (data not shown). To induce Th1 and Th2 polarized GA-reactive lines, PBMC cultures were treated with either IL-12 (2.5 ng/ml) and anti-IL-4 blocking antibody (2.5 mg/ml), or IL-4 (50 ng/ml) and anti-IL-12 blocking antibody (5 mg/ml), respectively, concurrent with the GA stimulation. Supernatants were collected from GA-reactive Th1 and Th2-polarized lines and unpolarized controls that had undergone at least three cycles of antigen stimulation. IFN γ and IL-5 were measured by sandwich ELISA as prototype Th1 and Th2 cytokines, using commercially available kits (Becton Dickinson, Franklin Lakes, NJ) as per the manufacturer's instructions.

Tissue culture – glial cells

Postmortem human CNS tissue was obtained from the Human Tissue Repository at the Albert Einstein College of Medicine, and all procedures were approved by the Institutional Clinical Review Committee. Spinal cord cultures enriched for oligodendrocytes were prepared as previously reported (Wilson et al., 2003). Rodent OPC cultures were prepared from P1 rat cortices by immunopanning for A2B5⁺ Ran2⁻ cells and were propagated or differentiated as previously described (Caporaso and Chao, 2001; Zhang et al., 2009).

Antibodies

The A2B5 mouse hybridoma was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and its antibody (IgM) prepared using standard methods. Other antibodies utilized included: O4 mouse monoclonal IgM (Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY); monoclonal mouse anti-CNPase (IgG1) and rabbit polyclonal anti-Olig2 (Chemicon, Temecula, CA); rabbit anti-GFAP (Invitrogen Zymed, Carlsbad, CA); mouse anti-MBP (IgG1) (Covance Sternberger, Berkeley, CA); mouse anti-IGF2 blocking antibody (R&D Systems, Minneapolis, MN). Previous work has established that the blocking anti-IGF-2 antibody used in our studies does not crossreact with IGF-1 (see <http://www.rndsystems.com/pdf/mab292.pdf>).

Immuofluorescence

For staining of tissue cultures, cells were fixed 10 min with 4% paraformaldehyde (PFA) at times specified, and processed for single- or double-immunostaining for A2B5 (1:15), O4 (1:25), CNPase (1:100), MBP (1:100), Olig-2 (1:100) and GFAP (1:100) using combinations described in the text. Immunostaining was carried out as previously described (Zhang et al., 2009; Zhang et al., 2006). Immunostained cultures were examined and photographed using a Zeiss LSM 510 META laser scanning confocal system attached to an Axiovert 200 inverted fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY). Z-series projections were collected and assembled using 1 μ m on the Z-axis using Zeiss LSM Image Browser software. Computer-assisted morphometric analysis was performed on random fields (at least five per condition) using ImageJ 1.30v software (NIH, Bethesda, MD), and results subjected to statistical analysis (see below).

IGF-2 ELISA

A sandwich ELISA kit for human IGF-2 was purchased from Diagnostic Systems Laboratories (Webster, TX) and used as per the manufacturer's instructions.

Microarray Studies

Microarray experiments were carried out as previously described (Mayne et al., 2001). A defined set of 1151 cDNAs was PCR amplified and printed on nylon filters by using a GMS417 Microarrayer (Genetic Microsystems). Clones were selected from a commercially available (Research Genetics, Inc.) master set of 15,000 human, verified-sequence, T1 phage-negative IMAGE Consortium clones. Total RNA was prepared from GA-reactive T cell lines prepared as above and polarized towards a Th1 or Th2 phenotype or left unpolarized (control). Total RNA was primed using oligo (dT) oligonucleotide (Research Genetics), and first-strand cDNA labeled with α -33P (3,000 Ci/mmol; ICN). Radioisotope incorporation was determined by counting a 1 μ l sample, and then probes were denatured and added to a microarray that had been pretreated for 4 h at 50°C with microhybridization buffer (Research Genetics). Hybridization continued overnight at 50°C, then microarrays were washed and exposed to phosphorimaging sheets (Storm) for 48 h and analyzed with a Storm PhosphorImager (Molecular Dynamics). All genes produced a quantifiable signal following hybridization.

Microarray Data Analysis

Gene expression was determined by capturing the pixel density of each 33P-labeled cDNA element using QuantOne Image Software (Bio-Rad). Raw intensity data for each experiment was normalized by Z transformation as previously described (Mayne et al., 2001). Briefly, first the raw pixel density data for each gene was $\log_{10}$ transformed and Z scores calculated by subtracting the average gene intensity (calculated from the sum of all data points) from the log intensity value of each gene and dividing that result by the standard deviation of all of the measured intensities. Z scores transform raw intensity data into a value for each individual gene reported as a function of its distance from the mean of all measured gene intensities, expressed in units of standard deviation. This facilitates comparisons between microarray experiments by adjusting for differences in hybridization intensity. Gene expression differences between experiments were calculated from the difference between the observed gene Z scores. Z ratios express differences in terms of their relationship to the standard deviation of the distribution of all of the observed gene changes. Calculated Z ratios for given comparisons were rank ordered on this basis.

Cluster analysis and statistics

Clustering of changes in gene expression was determined by using public domain Cluster software based on pairwise average-linkage cluster analysis (Eisen et al., 1998). Gene expression raw data, log values, and Z scores were averaged using mean $\pm$ standard deviation. Comparisons between experiments were conducted by regression analysis and Bonferroni's correction for multiple comparisons. For all tests, statistical significance was considered to be at the $p < 0.05$ level (InStat2; GraphPad Software, San Diego, Calif.). For experiments using OPCs or oligodendrocyte-enriched spinal cord cultures, statistical analysis was carried out using Prism software v4.0 (GraphPad). For multiple comparisons, ANOVA plus Bonferroni post test was used. For comparisons between two groups we used Student's t test, and $p < 0.05$ was considered significant.

3. Results

Supernatants from GA-reactive Th2-polarized lines exert trophic effects on rodent OPCs

To determine whether GA-reactive T cells regulate oligodendrocyte lineage cell number and/or differentiation, we initially exposed purified rodent OPC cultures to supernatants from Th1- or Th2-polarized GA-reactive lines from healthy human adult volunteers (Figures 1–3). In subsequent experiments, we confirmed relevance to our own species using primary

human oligodendrocyte-enriched cultures (Figure 4), and also explored underlying mechanisms using a combination of cDNA microarray analyses and functional studies (Figure 5). Lines of GA-reactive T lymphocytes were generated from peripheral blood of healthy adults as previously described (Kim et al., 2004a), and either polarized towards a Th1 phenotype using anti-IL-4 antibody and IL-12, towards a Th2 phenotype using IL-4 and anti-IL-12 antibody, or left unpolarized. Supernatants were collected from lines undergoing at least three cycles of GA stimulation, and IFN γ and IL-5 measured as prototype Th1 and Th2 cytokines by ELISA (Figure 1).

In initial experiments, supernatants from Th1- or Th2-polarized GA-reactive lines were added to the culture medium of purified rat OPCs, which were prepared from P1 cerebral cortices as previously described (Caporaso and Chao, 2001) (Figure 2). OPCs were plated into growth factor-free differentiation medium containing 33% supernatant or matched autologous serum-containing control, or proliferation medium containing 10ng/ml PDGF-AA and 10ng/ml FGF-2 plus supernatant or control. At 5d, cultures were fixed and stained for Olig2 (oligodendrocyte lineage), A2B5 (OPCs), O4, CNPase (mature oligodendrocytes), and GFAP (astrocytes), and counterstained with DAPI. Cultures were visualized by confocal microscopy and cells per random field (five 20x fields) were counted and compared statistically (ANOVA plus Bonferroni post test).

These studies showed that GA-reactive T lymphocytes produce soluble factors that result in trophic effects on oligodendrocyte lineage cells. OPC cultures left to differentiate for 5d in the presence of control autologous serum-containing medium contained Olig2⁺ oligodendrocyte lineage cells and GFAP⁺ astrocytes. The Olig2⁺ population comprised approximately $66.3 \pm 1.6\%$ total cells, and was composed of bipolar A2B5⁺ OPCs ($21.8 \pm 6.6\%$ of total cells), O4⁺ oligodendrocytes ($10.9 \pm 1.6\%$) and mature arborized CNPase⁺ cells ($48.3 \pm 9.8\%$) (Fig. 2a). The percentage of astrocytes was approximately $29.2 \pm 3.2\%$ of total cells, higher than in cultures grown in serum-free differentiation medium alone (5–10%), most likely due to the presence of serum in the culture medium. Importantly, cultures exposed to GA-reactive Th1- or Th2-polarized supernatants for 5d contained significantly more total (DAPI⁺) cells than autologous medium controls (Figs. 2b, 2c, Th1-polarized **p<0.01, Th2-polarized ***p<0.001 versus control, ANOVA plus Bonferroni post test). This effect was also associated with significant increases in absolute numbers and percentage of Olig2⁺ oligodendrocyte lineage cells (Figs. 2b, 2d, Th1 *p<0.05, Th2 **p<0.01) and O4⁺ oligodendrocytes (Figs. 2b, 2e, Th1 **p<0.01, Th2 *p<0.05) as well as CNPase⁺ mature oligodendrocytes (see Figure 3). Effects on GFAP⁺ astrocytes were more heterogeneous, although in most experiments astrocytes were significantly decreased after 5d in terms of absolute numbers and percentage of total cells (Fig. 2f, Th1 *p<0.05, Th2 **p<0.01). Effects of supernatants from Th2-polarized and Th1-polarized GA-reactive lines were generally comparable (Figs. 2c-f), although Th2-polarized supernatants had stronger effects than Th1-polarized lines on numbers of CNPase⁺ cells (Fig. 3b, GA-reactive Th2 versus Th1 p<0.001, ANOVA plus Bonferroni post test). Effects of GA-reactive supernatants were noticeably weaker in OPC cultures grown for 5d in proliferation medium in the presence of the mitogens PDGF-AA and FGF-2, reaching significance only in terms of total cell number, and only for Th2-polarized supernatants (*p<0.05) (Fig. 2g).

Collectively, these data showed that supernatants from GA-reactive T lymphocytes exert trophic effects on primary oligodendrocyte lineage cells.

Specificity of effects of GA-reactive T cell lines on OPC cultures

To examine the specificity of effects of GA-reactive T cell supernatants, we compared results from Th1- and Th2- polarized GA-reactive lines to data from Th1- and Th2-polarized OKT3-stimulated control lines from healthy adults (Figure 3). In place of selection

for GA reactivity, control lines were generated using polyclonal stimulation with OKT3 monoclonal antibody, which binds to the epsilon subunit within the human CD3 complex (Van Wauwe et al., 1980). To control for potential quantitative differences in stimulus intensity reflective of differential strength of stimulation, versus qualitative differences in supernatants generated using OKT3 versus GA, we titrated GA and OKT3 activation in these experiments such that they produced similar levels of T cell proliferation as assessed by CFSE dilution or ^3H -thymidine incorporation (data not shown).

OPC cultures were purified and plated into differentiation medium containing 33% supernatant from Th1- or Th2-polarized GA-reactive lines, OKT3-stimulated controls, or matched autologous serum-containing medium. After differentiation for 5d, cultures were fixed, stained for lineage and differentiation markers, and imaged by confocal microscopy. These experiments showed that the effects of supernatants from GA-reactive T cell lines on OPCs were significantly more potent than those of control OKT3-stimulated lines. OPC cultures exposed to GA-reactive Th1- or Th2-polarized cell supernatants contained significantly more total cells than their OKT3-stimulated supernatant counterparts (Fig. 3a, Th1, $p < 0.001$; Th2, $p < 0.01$, ANOVA plus Bonferroni post test), and significantly more CNPase⁺ oligodendrocytes (Fig. 3b, Th1, $p < 0.01$; Th2, $p < 0.001$). Effects of OKT3-stimulated T cell media were weak and reached significance over autologous serum-containing control medium only in the case of CNPase⁺ oligodendrocytes (Th2, $p < 0.05$), although CNPase⁺ cells comprised a similar percentage of total cells in OPC cultures exposed to both GA-reactive and OKT3-stimulated samples (Fig. 3c). Collectively, these data reveal that the trophic effects of GA-reactive T lymphocyte lines on OPC cultures are distinct from any effects resulting from nonspecific T cell activation.

Trophic effects of supernatants from GA-reactive Th2-polarized lines on primary human oligodendrocytes *in vitro*

To determine the relevance of these findings to our own species, we examined effects of human GA-reactive T cell supernatants on primary oligodendrocyte-enriched cultures from human spinal cord samples (Figure 4). These cultures do not yield cells in sufficient numbers to allow for routine purification by immunopanning, and produce human oligodendrocyte lineage cells of all differentiation states (Zhang et al., 2006). Cultures were established as previously reported (Wilson et al., 2003), and plated into differentiation medium containing 33% Th1 or Th2 supernatant from GA-reactive T lymphocytes or autologous serum-containing medium control. At 5d, cultures were fixed and stained for lineage and differentiation markers as above. Cells per random field (five 20x fields) were counted and compared.

These studies confirmed that supernatants from human GA-reactive T lymphocytes have trophic effects on human oligodendrocytes. Spinal cord cultures left to differentiate for 5d in the presence of control autologous serum-containing medium contained Olig2⁺ oligodendrocyte lineage cells and GFAP⁺ astrocytes. Olig2⁺ cells comprised only $26.7 \pm 3.6\%$ total cells, compared with approximately 75% of total cells in similar cultures grown in serum-free differentiation medium (Zhang et al., 2006). Importantly, cultures exposed to GA-reactive Th2-polarized cell supernatants for 5d displayed a significant increase in total cell number (Figs. 4a,b, $**p < 0.01$, ANOVA plus Bonferroni post test), and this was also associated with an increase in both the absolute number and percentage of Olig2⁺ oligodendrocyte lineage cells (Fig. 4c, $**p < 0.01$). In cultures exposed to Th2-polarized supernatants, the percentage of Olig2⁺ cells increased to $51.1 \pm 3.6\%$ of total cells (Fig. 4c). Supernatants from Th1-polarized lines reached significance only in terms of an increase in the percentage of Olig2⁺ cells (Fig. 4c, $*p < 0.05$, $46.3 \pm 6.3\%$ cells). Collectively, these data establish that supernatants from human GA-reactive T cell lines have trophic effects on

human oligodendrocyte lineage cells, and were compatible with our findings from purified rodent OPC cultures.

GA-reactive Th2-polarized lines display induction of neurotrophic genes

To examine the mechanism underlying these effects, we analyzed transcriptional patterns in GA-reactive Th1- and Th2-polarized T cell lines using microarray analysis (Figure 5). First-strand cDNA from GA-reactive T cell lines polarized towards a Th1 or Th2 phenotype, or unpolarized control, was labeled with α -33P, and probes denatured and hybridized to a nylon filter microarray containing a defined set of 1151 cDNAs (see Methods section) (Mayne et al., 2001). Raw intensity data for each element was normalized by Z transformation (Mayne et al., 2001), and Z scores were subjected to cluster analysis (Eisen et al., 1998). Z ratios were generated for all elements including Th1- and Th2-polarized lines versus unpolarized controls. Gene expression raw data, log values, and Z scores were averaged and compared by regression analysis and Bonferroni's correction for multiple comparisons.

These studies showed that GA-reactive T cell lines express factors previously implicated as regulators of survival, proliferation and/or differentiation of oligodendrocyte lineage cells (Figure 5a). Interestingly, induced or upregulated transcripts included factors not previously reported as expressed by GA-reactive lymphocytes. Th1- and Th2-polarized lines displayed differential expression patterns. Z ratio data indicated that transcripts induced in Th2-polarized lines included IGF-2, CXCL1, FGF1, EDN1, LIF and BMP4 (Fig. 5a). Insulin-like growth factor-2 (IGF-2) has previously been reported to act as a trophic factor for OPCs similar to IGF-1 (Nicholas et al., 2002), while fibroblast growth factor-1 (FGF-1, acidic FGF) and the CXC chemokine CXCL1/GRO1 are OPC mitogens (Robinson et al., 1998; Vick and DeVries, 1992; Wu et al., 2000). Endothelin-1 (EDN-1) regulates OPC migration (Gadea et al., 2008), whereas leukemia inhibitory factor (LIF) is a survival factor for the oligodendrocyte lineage (Butzkueven et al., 2002). In contrast, bone morphogenetic protein-4 (BMP-4) restricts OPC maturation and is an important determinant of the positional distribution of OPC development in embryonic spinal cord (Miller et al., 2004). Z ratio data showed that transcripts induced in Th1-polarized lines included fewer known regulators of the oligodendrocyte lineage (Fig. 5a). Upregulated genes included follistatin (FST) as well as chemokine (C-X-C motif) ligand 3 (CXCL3) and bone morphogenetic protein 7 (BMP7). Follistatin is an inhibitor of BMPs and activins, which also regulate OPC differentiation (Zameer et al., submitted). CXCL3 has not been directly implicated as a regulator of the oligodendrocyte lineage, but is a ligand for CXCR2 and related to CXCL1, both of which are known to regulate OPC proliferation and/or migration (Miller et al., 2004). BMP7 has been suggested as having properties similar to BMP4 (Gao et al., 2006). To determine the significance of these findings, we subjected data to regression analysis incorporating Bonferroni's correction for multiple comparisons (Fig. 5a). The results of these studies showed that induction of IGF-2 was strongly significant in Th2-polarized lines (p value 9.24×10^{-10}), while induction of BMP7 reached significance in Th1-polarized lines (p value 0.0002). Induction of other factors did not reach statistical significance (Fig. 5a).

Collectively, these data suggested that GA-reactive T lymphocytes produce regulatory factors for oligodendrocyte lineage cells beyond those already reported, including IGF-2 and BMP7.

GA-reactive Th2-polarized lines produce IGF-2, a trophic factor for oligodendrocyte lineage cells

To test the functional relevance of these findings, we examined the expression and functional properties of IGF-2, which our microarray data suggested was upregulated in GA-

reactive Th2-polarized lines (Z ratio 7.16, p value 9.24 E-10). To determine whether induction of IGF-2 at the mRNA level was reflected in changes in IGF-2 protein, we carried out ELISA studies (Fig. 5b). IGF-2 concentrations were measured in supernatants from GA-reactive Th1- and Th2-polarized cells and autologous serum-containing and serum-free control media, using a commercially available assay. IGF-2 was also measured in OPC differentiation medium, and in conditioned medium from OPC cultures. These experiments revealed that control serum-free T cell medium did not contain detectable IGF-2, but that concentrations of approximately 830pg/ml IGF-2 were present in medium containing 5% autologous serum. Levels of IGF-2 were further increased in supernatants from Th1- and Th2-polarized GA-reactive lymphocytes, and in the case of Th2-polarized cells this increase reached significance (Fig. 5b, $p < 0.05$, ANOVA plus Bonferroni post test, 1150pg/ml). Neither OPC differentiation medium nor conditioned medium from OPC cultures contained detectable IGF-2 (data not shown).

To determine the functional significance of IGF-2 to oligodendrocyte lineage cell cultures *in vitro*, we carried out studies in the presence of a commercially available blocking antibody specific for human IGF-2 (R&D Systems) (Fig. 5c). Previous work using direct ELISA and immunoblotting has confirmed that this antibody shows no cross-reactivity with recombinant human or mouse IGF-1 (see Methods section). Supernatants from GA-reactive Th2-polarized lines or autologous serum-containing medium controls were preincubated with 50µg/ml anti-IGF-2 or IgG control for 1h, then antibodies removed by centrifugation for 5min at 20,000G, and supernatants added to purified OPC cultures as above. At 5d, cultures were fixed, stained for lineage and differentiation markers and visualized as above. These experiments confirmed IGF-2 as a trophic factor for oligodendrocyte lineage cells *in vitro*. OPC cultures exposed to GA-reactive supernatants preincubated with blocking anti-IGF-2 antibody displayed a 30% reduction in the numbers of both total cells (DAPI⁺) and Olig2⁺ oligodendrocyte lineage cells, and in both cases these changes were highly significant (Fig. 5c, DAPI, $p < 0.01$; Olig2⁺, $p < 0.001$, Student's t test). Moreover, compatible with the results of our ELISA studies showing IGF-2 in autologous serum-containing medium, preincubation of serum-containing medium with blocking antibody also resulted in a similar decrease in cell numbers (data not shown). As an additional condition to control for potential carry-over effects of antibodies in OPC cultures, in some experiments cultures of OPCs in differentiation medium were treated with the same concentration of anti-IGF2 or IgG, and results measured. In the absence of IGF-2-containing supernatants, anti-IGF-2 antibody had no effect on OPC numbers or maturation (data not shown).

Collectively, these data affirm that IGF-2 is present in the serum of healthy adult volunteers, and that it is produced at significantly higher concentrations by GA-reactive Th2-polarized human lymphocytes. Our findings implicate IGF-2 as a trophic factor for oligodendrocyte lineage cells, and suggest it as one of the group of mediators contributing to the effects of GA-reactive Th2 cells on OPC cultures *in vitro*, and perhaps *in vivo* within the human CNS.

4. Discussion

In RRMS patients, binding of GA to MHC class II molecules is believed to inhibit binding of MBP and activation of encephalitogenic T lymphocytes (Fridkis-Hareli et al., 1999; Fridkis-Hareli and Strominger, 1998). GA-reactive T cells are also known to be present in the circulation of normal individuals, and are predominantly of a Th2 phenotype (Aharoni et al., 1997; Chen et al., 2002; Dhib-Jalbut et al., 2003; Neuhaus et al., 2000). Activation of these cells suppresses the activity of autoaggressive myelin-specific T helper 1 (Th1) lymphocytes (Aharoni et al., 1998), and this is believed to be an important mechanism underlying the beneficial effects of GA in EAE. GA treatment also induces type 2 myeloid

cell differentiation with consequent Th2 T cell differentiation (Kim et al., 2004b; Weber et al., 2004).

In counterpoint to its widely-acknowledged immunoregulatory effects, recent findings have suggested an additional neurotrophic role for GA therapy in inflammatory demyelinating disease, and the results of our study support this hypothesis. Our data are compatible with recent work from other groups showing that GA-reactive T lymphocytes produce factors trophic for the oligodendrocyte lineage, including IGF-1 and BDNF (Aharoni et al., 2005; Chen et al., 2003; Skihar et al., 2009; Ziemssen et al., 2002). IGF-1 is a growth and differentiation factor for OPCs (Hsieh et al., 2004) that binds to the tyrosine kinase receptor IGF1R and activates PI3K-Akt signaling. IGF-1 also increases CNS myelination in transgenic models (Carson et al., 1993). The neurotrophin BDNF activates the tyrosine kinase receptor TrkB and has been shown to potentiate CNTF-induced oligodendrocyte survival *in vitro* (Barres et al., 1993). GA treatment of EAE is also associated with elevated CNS levels of the neurotrophins NT3 and NT4 (Aharoni et al., 2005). Mice lacking functional NT3 or its receptor TrkC are deficient in both OPCs and mature oligodendrocytes (Kumar et al., 1998). Thus, although the capacity to produce neurotrophins is not unique to GA-reactive T lymphocytes (Stadelmann et al., 2002), these previous results have suggested that factors produced by GA-reactive T lymphocytes may exert supportive or instructive effects on oligodendrocyte lineage cells in inflammatory demyelinating lesions. This hypothesis is further supported by recent *in vivo* findings demonstrating that in mice subjected to lyssolecithin-induced demyelination of the spinal cord, GA-treated animals display greater numbers of OPCs in the demyelinated dorsal column at 7dpi and more extensive remyelination at 28dpi compared with controls. This difference is further associated with elevated levels of IGF-1 and BDNF in the spinal cord of the GA-treated mice (Skihar et al., 2009).

The findings of our experiments suggest that IGF-1 and members of the neurotrophin family are not the only factors produced by human GA-reactive lymphocytes that are trophic for oligodendrocyte lineage cells. In purified cultures of rodent OPCs we found that supernatants from human GA-reactive lines induced significant increases in numbers of total cells and Olig2⁺, O4⁺ and CNPase⁺ oligodendrocytes (Figures 2,3). Effects of supernatants from Th2-polarized and Th1-polarized GA-reactive lines were generally comparable in rodent OPC cultures (Figure 2), although Th2-polarized supernatants had stronger effects than Th1-polarized lines on numbers of CNPase⁺ cells (Figure 3). The effects of supernatants from GA-reactive lines were significantly more potent than those from OKT3-stimulated controls (Figure 3). Importantly, we also observed similar effects in human oligodendrocyte-enriched cultures, in which supernatants from human derived GA-reactive Th2 lines were associated with significant increases in the number of total cells, and both Th1 and Th2 lines induced significant increases in Olig2⁺ oligodendrocyte lineage cells (Figure 4). In order to explore underlying mechanisms, we analyzed microarray data of transcriptional patterns in GA-reactive T lymphocytes (Figure 5). We found that multiple trophic factors for oligodendrocyte lineage cells were induced or upregulated in Th2-polarized GA-reactive T cell lines, including IGF-2, FGF-1, EDN-1 and LIF. Notably, expression of these factors by GA-reactive lymphocytes has not been reported previously. Statistical analysis of these results showed that induction of IGF-2 was strongly significant (p value 9.24 E-10) in Th2-polarized lines, while induction of other factors did not reach statistical significance. IGF-2 has been shown to potentiate the growth and differentiation of OPCs *in vitro* (Nicholas et al., 2002). To validate our microarray data, we carried out sandwich ELISA analyses of IGF-2 protein concentrations in GA-reactive T cell supernatants, and these experiments confirmed the presence of IGF-2 in serum-containing medium and its upregulation in Th2-polarized GA-reactive lines (Figure 5). By contrast, neither OPC differentiation medium nor conditioned medium from OPC cultures contained

detectable IGF-2. Importantly, suggesting functional relevance of our finding, blockade of IGF-2 significantly inhibited the trophic effects of supernatants from GA-reactive Th2-polarized lines on OPC cultures. Previous work has shown that the IGF-2 antibody used in these experiments does not crossreact with IGF-1 (see Methods section). Collectively, our data suggest that GA-reactive T cells produce multiple factors that regulate the survival, proliferation or maturation of oligodendrocyte lineage cells. Specifically, our results identify a role for IGF-2 in the effects of GA. In concert with the work of Skihar *et al.*, they emphasize members of the insulin-like growth factor family as downstream effectors of GA treatment in oligodendrocyte lineage cells.

Our findings are compatible with the concept that inflammation may regulate the capacity for repair in affected tissues positively as well as negatively, depending on the nature of the immune response. While the inflammatory response in acute MS lesions is classically associated with myelin destruction (Raine *et al.*, 1997), conversely repair of demyelinating lesions is restricted following depletion of either mononuclear phagocytes or CD4⁺ or CD8⁺ T lymphocytes (Bieber *et al.*, 2003; Kotter *et al.*, 2001; Neumann *et al.*, 2009). Potentiation of inflammation may also lead to increased remyelination in some demyelinating models (Foote and Blakemore, 2005; Setzu *et al.*, 2006). Additionally, while the persistence of low-grade inflammation in more chronic MS lesions has been linked to progressive axonal transection and neuronal loss (Trapp *et al.*, 1998), it has also been shown that introduction of T lymphocytes specific for a CNS self-antigen such as myelin basic protein can protect damaged neurons from secondary degeneration in models of neuronal injury (Hammarberg *et al.*, 2000; Moalem *et al.*, 1999). Mechanisms proposed to contribute to these positive roles of inflammation include production of neurotrophic and/or neuroprotective factors by immune cells, and expression of growth and chemotactic factors for the proliferation and recruitment of OPCs that may facilitate remyelination (Kerschensteiner *et al.*, 2009; Kotter *et al.*, 2001). In addition to its widely acknowledged role in diverting the immune response away from production of activated encephalitogenic lymphocytes, our data suggest that GA therapy results in production by GA-reactive T lymphocytes (particularly Th2-polarized cells) of factors that are directly trophic for the oligodendrocyte lineage. Thus, in addition to diminishing the negative effects of inflammation, GA therapy may accentuate its positive actions.

Our results may also be instructive in a more basic context. While the concept of therapeutic design to enhance neuroprotection and repair in MS is attractive, approaches using administration to human patients of single recombinant factors designed to modulate oligodendrocyte survival and regeneration have not produced strong results in clinical trials. While use of single recombinant factors is a highly focused approach, a cohort of mediators with complementary or perhaps synergistic effects may represent an option with a higher chance of success, however identifying potentially effective combinations is difficult and complex. Our work and those of other groups show that Th2 lines reactive for GA produce multiple factors known to be trophic for the oligodendrocyte lineage, and importantly, suggest the functional relevance of these effects. Further study of the properties of these cells may be a useful tool to facilitate our understanding of how different growth factors interact to potentiate oligodendrocyte survival and maturation, and enhance myelin protection and repair in demyelinating lesions.

In conclusion, while research into the effects of GA in RRMS has focused primarily on immune modulation, recent work suggests an additional neurotrophic mechanism of action. Our findings support this hypothesis, and show that trophic factors produced by GA-specific T lymphocytes impact the numbers of oligodendrocyte lineage cells *in vitro*. Moreover, our data indicate that the collection of factors produced by these cells is more extensive than previously reported, and specifically identify a role for at least one new factor, IGF-2, in

these effects. Collectively, our results are compatible with the concept that in addition to its well-characterized immunoregulatory actions, GA may exert supportive effects on oligodendrocyte lineage cells in patients with RRMS.

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GA		
D-32		
pTh1	IFN-g	IL-5
Line#		
1	5945	0
2	5815	0
3	5301	2486
4	6364	0
5	7059	0
6		
7	6716	0
8	6433	787
9	6785	0
10	7114	1180
11	5364	0
12	6014	741

D-32		
pTh2	IFN-g	IL-5
Line#		
1	763	6708
2		
3	887	5606
4		
5		
6	0	5412
7	0	5724
8		

D-32		
NP	IFN-g	IL-5
Line#		
1	1660	0
2	5413	0
3	3893	0
4	2417	0
5	2948	0
6	2378	1205
7	3206	2826
8	5721	673
9	5771	4625

Figure 1. Generation of supernatants from GA-reactive polarized Th1 and Th2 lines

Data shown illustrate the cytokine profiles (pg/ml) of individual GA-reactive T lymphocyte lines from healthy adult controls, polarized for a Th1 or Th2 phenotype (pTh1, pTh2) or nonpolarized (NP), with supernatants removed and assayed at 32 days in culture (D-32). To generate GA-reactive T cell lines from peripheral blood of healthy adults, PBMC were plated in medium containing 10ng/ml IL-7 without antigen, or with 20µg/ml GA. After 7d, medium was enriched with rhIL-2 (20U/ml), and wells were evaluated for GA specificity at 11d. GA-reactive wells (stimulation index >3, and proliferation >3 SD vs. control) were expanded as lines with rhIL-2, and restimulated with GA and autologous irradiated PBMCs. To produce Th1 and Th2 polarization, lines were treated with IL-12 (2.5ng/ml) and anti-IL-4

antibody (2.5mg/ml), or IL-4 (50ng/ml) and anti-IL-12 antibody (5mg/ml) concurrent with GA stimulation. Supernatants were collected from individual GA-reactive Th1 or Th2 polarized and unpolarized control lines undergoing at least three cycles of antigen stimulation. IFN γ and IL-5 were measured as Th1 and Th2 cytokines by sandwich ELISA.

Figure 2. Supernatants from GA-reactive Th2-polarized lines exert trophic effects on rodent OPCs

Primary OPC cultures were purified from P1 rat cortices and plated in differentiation medium (no growth factors) or proliferation medium (+10 ng/ml PDGF-AA +10 ng/ml FGF-2) containing 33% supernatant from GA-reactive Th1- or Th2-polarized T cell lines, or matched autologous serum-containing medium control. **(a)** After 5d, cultures contained Olig2⁺ oligodendrocyte lineage cells and GFAP⁺ astrocytes. Olig2⁺ cells could be subdivided into bipolar A2B5⁺ OPCs, O4⁺ oligodendrocytes, and mature arborized CNPase⁺ cells. **(b-f)** Cultures in basic medium exposed to GA-reactive Th2-polarized cell supernatants for 5d contained more total cells than controls (**b,c**, $p < 0.001$, ANOVA plus Bonferroni post test). This effect was associated with significant increases in the absolute numbers and percentage of Olig2⁺ cells (**b,d**, $p < 0.01$),⁺ oligodendrocytes (**b,e**, $p < 0.05$), and decreased numbers and percentage of GFAP⁺ astrocytes (**f**, $p < 0.01$). Effects of Th1-polarized lines also reached significance in all cases (**c-f**, P values shown). **(g)** In OPC cultures grown in proliferation medium, GA-reactive T cell supernatants had weaker effects, which reached significance only in terms of total cell number, and only for Th2-polarized cells ($p < 0.05$). **(c-g)** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scalebars, **(a)** 20 μ m, **(b)** 50 μ m. Data are representative of at least three experiments in independent cultures.

Figure 3. Specificity of effects of GA-reactive T cell lines on OPC cultures

Rodent P1 OPCs were plated into differentiation medium (no growth factors) containing 33% supernatant from GA-reactive lines, OKT3-stimulated control lines, or autologous serum-containing medium. Strengths of GA stimulation and OKT3 stimulation were titrated to produce similar levels of T cell proliferation as assessed by CFSE dilution or tritiated thymidine incorporation (data not shown). **(a,b)** After maturation for 5d, OPC cultures exposed to GA-reactive Th1- or Th2-polarized cell supernatants contained significantly more total cells than their OKT3-stimulated supernatant counterparts (**a**, Th1, $p < 0.001$; Th2, $p < 0.01$ ANOVA plus Bonferroni post test), and significantly more CNPase⁺ oligodendrocytes (**b**, Th1, $p < 0.01$; Th2, $p < 0.001$). Effects of OKT3-stimulated T cell media were weak and reached significance over autologous serum-containing control medium only in the case of CNPase⁺ cells (**b**, Th2, $p < 0.05$), although interestingly, CNPase⁺ oligodendrocytes comprised a similar percentage of total cells in OPC cultures exposed to both GA-reactive and OKT3-stimulated samples (**c**). **(a-c)** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ANOVA plus Bonferroni post test. Data shown are representative of at least three experiments using independent cultures.

Figure 4. Trophic effects of supernatants from GA-reactive Th2-polarized lines on primary human oligodendrocytes *in vitro*

Primary human oligodendrocyte-enriched cultures prepared from spinal cord samples were plated into differentiation medium containing 33% supernatant from Th1- or Th2-polarized GA-reactive T lymphocytes or autologous serum-containing medium control. **(a,b)** Cultures exposed to GA-reactive Th2-polarized cell supernatants for 5d displayed a significant increase in total cell number (** $p < 0.01$). **(a,c)** This effect was also associated with an increase in both the number and percentage of Olig2⁺ oligodendrocyte lineage cells (** $p < 0.01$). Supernatants from Th1-polarized lines had weaker effects, which reached significance only in terms of an increase in the percentage of Olig2⁺ cells (**c**, * $p < 0.05$). **(b,c)** * $p < 0.05$, ** $p < 0.01$, ANOVA plus Bonferroni post test. Scale bar, (a), 30 μ m. Data are representative of at least three experiments using independent cultures.

Figure 5. GA-reactive Th2-polarized lines produce IGF-2, a trophic factor for oligodendrocyte lineage cells

(a) First-strand cDNA from Th1- or Th2-polarized GA-reactive T cell lines or unpolarized controls was labeled with α -33P and hybridized to a nylon filter microarray containing 1151 cDNAs. The pixel density of each 33P-labeled cDNA element was determined and raw intensity data normalized for each element by Z transformation. Gene expression raw data, log values, and Z scores were averaged using mean $\pm$ SD and compared by regression analysis and Bonferroni's correction for multiple comparisons. Z score data indicated that GA-reactive lines express multiple factors implicated as regulators of the growth and/or differentiation of oligodendrocyte lineage cells, including factors not previously identified as expressed by GA-reactive cells. Transcripts induced in Th2-polarized lines included IGF-2, CXCL1, FGF1, EDN1, LIF and BMP4, while transcripts induced in Th1-polarized lines included FST, CXCL3 and BMP7. Statistical analysis showed that induction of IGF-2 was strongly significant (p value 9.24 E-10) in Th2-polarized lines, while induction of BMP7 reached significance in Th1-polarized lines (p value 0.0002). Other factors did not reach statistical significance. (b) To determine whether induction of IGF-2 was reflected in changes in IGF-2 protein, supernatants from GA-reactive Th1- and Th2-polarized cells and 5% autologous serum-containing and serum-free control media were analyzed by ELISA. IGF-2 was detected in medium containing autologous serum at concentrations of approximately 830 pg/ml, and concentrations were further increased in supernatants from Th1- and Th2-polarized GA-reactive lymphocytes. In the case of Th2-polarized cells this increase reached significance (*p<0.05, ANOVA plus Bonferroni post test, 1150 pg/ml). IGF-2 was not detected in serum-free control medium, nor in OPC differentiation medium or OPC-conditioned medium (data not shown). (c) Supernatants from GA-reactive Th2-polarized lines were preincubated with 50 μ g/ml anti-IGF-2 blocking antibody or IgG control at room temperature 1h, then added to purified OPC cultures as above. After 5d,

cultures exposed to supernatants preincubated with anti-IGF-2 antibody displayed a 30% reduction in numbers of both total cells (DAPI⁺) and Olig2⁺ oligodendrocyte lineage cells compared with controls, and in both cases these changes were significant (DAPI⁺ **p<0.01, Olig2⁺ ***p<0.001). In the absence of IGF-2-containing supernatants, anti-IGF-2 antibody had no effect on OPC numbers or maturation (data not shown). **(b,c)** *p<0.05, **p<0.01, ***p<0.001, ANOVA plus Bonferroni post test **(b)**, Student's t test **(c)**. Data shown are representative of at least three experiments using independent cultures.