3,3'-Diindolylmethane Ameliorates Experimental Autoimmune Encephalomyelitis by Promoting Cell Cycle Arrest and Apoptosis in Activated T Cells through MicroRNA Signaling Pathways

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ABSTRACT

3,3'-Diindolylmethane (DIM) is a naturally derived indole found in cruciferous vegetables that has great potential as a novel and effective therapeutic agent. In the current study, we investigated the effects of DIM post-treatment on the regulation of activated T cells during the development of experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis. We demonstrated that the administration of DIM 10 days after EAE induction was effective at ameliorating disease parameters, including inflammation and central nervous system cellular infiltration. MicroRNA (miRNA) microarray analysis revealed an altered miRNA profile in brain infiltrating CD4⁺ T cells following DIM post-treatment of EAE mice. Additionally, bioinformatics analysis suggested the involvement of DIM-induced miRNAs in pathways and processes that halt cell cycle progression and

promote apoptosis. Additional studies confirmed that DIM impacted these cellular processes in activated T cells. Further evidence indicated that DIM treatment significantly upregulated several miRNAs (miR-200c, miR-146a, miR-16, miR-93, and miR-22) in brain CD4⁺ T cells during EAE while suppressing their associated target genes. Similarly, we found that overexpression of miR-16 in primary CD4⁺ T cells led to significant down-regulation of both mRNA and protein levels of cyclin E1 and B-cell lymphoma-2, which play important roles in regulating cell cycle progression and apoptosis. Collectively, these studies demonstrate that DIM post-treatment leads to the amelioration of EAE development by suppressing T-cell responses through the induction of select miRNAs that control cell cycle progression and mediate apoptosis.

Introduction

Multiple sclerosis (MS) is characterized as a relapsing-remitting inflammatory and autoimmune disease in which activated autoreactive T cells migrate to the central nervous system (CNS) and trigger inflammation. As a result, conditions such as demyelination of neurons and intermittent episodes of neurologic dysfunction including visual impairment, ataxia, motor sensory deficits, and bowel and bladder incontinence may ensue (Siffrin et al., 2007). Experimental autoimmune encephalomyelitis (EAE) animal models have served as invaluable laboratory tools for examining the T cell-dependent pathogenesis of autoimmune inflammation and demyelination within the CNS of MS patients (Schreiner

et al., 2009). During the course of EAE, naïve T cells in secondary lymphoid organs are presented with self-myelin-based antigens. Upon activation, these T cells migrate across the blood-brain barrier and into the CNS, resulting in inflammatory lesions and development of disease (Dijkstra et al., 1992; Xiao et al., 1998). Thus, an effective therapeutic strategy would in part restrict T-cell function and responses subsequently leading to the amelioration of disease development.

MicroRNAs (miRNAs) are small (~22 nt), endogenous, noncoding RNA molecules that have recently been found to act as primary regulators of gene expression (Baek et al., 2008; Selbach et al., 2008). Several studies have demonstrated that miRNAs play critical roles in modulating the immune system and promoting the development of certain diseases (Lodish et al., 2008; Du et al., 2009; Junker et al., 2009; O'Connell et al., 2010a,b). For example, overexpression of miR-326 was found to exacerbate symptoms of EAE in mice and correlated with disease severity in multiple sclerosis patients (Du et al., 2009). Similarly, miR-155 is one of the primary miRNAs that are upregulated in the CNS of MS patients (O'Connell et al., 2010a). In contrast, the silencing of miR-326 and miR-155 in these studies resulted in diminished a T-cell

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ABBREVIATIONS: BMDC, bone marrow-derived dendritic cell; CNS, central nervous system; ConA, concanavalin A; DIM, 3,3'-diindolylmethane; DMSO, dimethylsulfoxide; EAE, experimental autoimmune encephalomyelitis; IFN γ , interferon- γ ; IPA, Ingenuity Pathway Analysis; miRNA, microRNA; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PE, phycoerythrin; qRT-PCR, quantitative real-time polymerase chain reaction; 3'-UTR, 3'-untranslated region.

number and responses in the CNS and consequently milder clinical symptoms.

The benefits of natural products have been observed in traditional medicines for centuries to treat a variety of diseases, such as arthritis (Cock and van Vuuren, 2014), multiple sclerosis (Grotenhermen and Müller-Vahl, 2012), and inflammatory bowel disease (Ng et al., 2013). More recently, the use of plant-derived indoles, such as 3,3'-diindolylmethane (DIM), which are found in cruciferous vegetables, and other similarly structured compounds, have been reported as a potential therapeutic modality against inflammatory disorders (Busbee et al., 2013). Data from various cancer studies, which include prostate (Nachshon-Kedmi et al., 2003), colon (Lerner et al., 2012), and gastric (Li et al., 2013) cancers, have collectively suggested that DIM also acts on a number of molecular pathways centered on cellular proliferation and survival to mediate its effects. In contrast, the immunomodulatory and antiproliferative properties of DIM have not been well studied, particularly in regards to autoimmune diseases.

In this study, we tested the hypothesis that DIM post-treatment can regulate T cell-mediated responses, a driving force behind EAE development, by modulating miRNA expression and thereby mitigating disease parameters. Our data indicate that treatment of EAE mice with DIM given 10 days after disease induction effectively curtailed the clinical disorder. During our investigation, we identified a number of miRNAs in brain infiltrating T cells that were altered by DIM post-treatment during EAE development. Furthermore, five of the top upregulated miRNAs were found to regulate genes that play key roles in restricting cell cycle progression and cell survival, particularly miR-16. Overall, our studies suggest that natural products, such as the plant-derived indole DIM, can be used to modulate critical miRNAs involved in cell cycle progression and apoptosis, thus hindering T-cell responses and limiting the development of EAE.

Material and Methods

Mice. Female C57BL/6 mice and C57BL/6-Tg (Tcr2D2, Tcrb2D2) 1Kuch/J mice (2D2) (6–8 weeks old) were purchased from the National Cancer Institute (Bethesda, MD).

Ethics Statement. All animals were housed at the Association for Assessment and Accreditation of Laboratory Animal Care-accredited University of South Carolina, School of Medicine (Columbia, SC). All animal procedures were performed according to National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee of the University of South Carolina.

Reagents and Monoclonal Antibodies. The reagents were purchased as follows: DIM, concanavalin A (ConA), red blood cell lysis buffer, β -mercaptoethanol, and corn oil from Sigma-Aldrich (St. Louis, MO); propidium iodide from Molecular Probes (Eugene, OR); RNase buffer from Invitrogen (Grand Island, NY); Percoll from GE Healthcare Life Sciences (Pittsburgh, PA); RPMI 1640, L-glutamine, HEPES, phosphate-buffered saline, and fetal bovine serum from VWR (West Chester, PA); and myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) peptide and H-MEVGWYRSPFSRVVHLYRNGK-OH from PolyPeptide Laboratories San Diego (San Diego, CA). In situ cell death detection and fluorescein kits (terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling) were purchased from Roche (Indianapolis, IN).

The following monoclonal antibodies were purchased from BioLegend (San Diego, CA): fluorescein isothiocyanate-conjugated anti-mouse CD4 (L3T4) monoclonal antibody (GK1.5; rat IgG2b), CD69 (H1.2F3); phycoerythrin (PE)-conjugated anti-mouse CD3e (145-2C11; hamster IgG);

allophycocyanin anti-mouse CD8 (Ly-2) (53-6.7; rat IgG2a), CD28 (37.51); PE-Cy7-conjugated anti-mouse CD25 (PC61); and affinity purified anti-mouse CD16/32 (93; rat IgG2a).

Effects of DIM on EAE in Mice. EAE was induced in female C57BL/6 mice (6–8 weeks old) via subcutaneous immunization with 100 μ l of 150 μ g MOG₃₅₋₅₅ peptide emulsified in complete Freund's adjuvant (Difco, Detroit, MI) containing 4 mg/ml killed *Mycobacterium tuberculosis* (strain H37Ra; Difco), as described by us previously (Singh et al., 2007; Rouse et al., 2013). Following immunization, 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) was injected intraperitoneally into mice on day 0, followed by a 400-ng pertussis toxin intraperitoneal injection on day 2. Mice were randomized and treated with vehicle [2% dimethylsulfoxide (DMSO) + corn oil] or 40 mg/kg DIM i.p. 10 days after the induction of EAE and this treatment continued every day throughout the remainder of the study. Such treatment with the indole was designated as "post-treatment." Animals were monitored and clinical scores were evaluated and recorded on a daily basis. The mean score was calculated for each group every day. The measured parameters were as follows: 0 = no symptoms; 1 = limp tail; 2 = partial paralysis of hind limbs; 3 = complete paralysis of hind limbs or partial hind and front limb paralysis; 4 = tetraparalysis; and 5 = moribund. Additional precautions were made available to paralytic animals to ensure their accessibility to fresh food and water, as well as medical intervention if animals were under distress outside of experimental conditions. Death was not used as an index for clinical scores, and any mice that were moribund were immediately euthanized using the inhalant anesthetic isoflurane, a method approved by the American Association for Laboratory Animal Science.

Histopathological Analysis. Spinal cords were isolated from naïve or EAE mice post-treated with vehicle or DIM 15 days after immunization. Tissue was fixed with 10% formalin and paraffin blocks were prepared. Microtome sections (10 μ m) were generated, and tissue sections were stained with H&E.

In Vitro Cell Culture Assays. Cell cultures were maintained in complete RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 10 mM L-glutamine, 10 mM HEPES, 50 μ M β -mercaptoethanol, and 100 μ g/ml penicillin/streptomycin at 37°C and 5% CO₂.

Analysis of Inflammatory Cytokines after MOG₃₅₋₅₅ Restimulation. Splenocytes isolated from naïve and EAE + vehicle mice were isolated 15 days after disease induction. Cells were restimulated with MOG₃₅₋₅₅ peptide (30 μ g/ml) and treated with either vehicle or the indicated doses of DIM for 3 days. Supernatants were collected and analyzed for interferon- γ (IFN γ) using a BioLegend enzyme-linked immunosorbent assay kit (San Diego, CA) according to the manufacturer's instructions.

Effects of DIM on T-Cell Activation. Primary T cells were isolated from lymph nodes and purified using nylon wool. The cells were confirmed to be 95% CD3⁺ T cells by flow cytometry. Cells were seeded at 1×10^6 cells/well in a 96-well plate and cultured as naïve or activated with ConA (2.5 μ g/ml). Vehicle (DMSO) or DIM (100 μ M) was added simultaneously to activated cells for 24 hours. Cells were harvested and stained for various T-cell activation markers and analyzed using flow cytometry in which samples were gated on live cells.

Differential Expression and Validation of DIM-Induced miRNAs during EAE and Associated Target Genes by Quantitative Real-Time Polymerase Chain Reaction. Mice induced with EAE were given vehicle or DIM as indicated. On day 15, mononuclear cells from pooled brain homogenates ($n = 5$ per group) were isolated using 33% Percoll. Subsequently, CD4⁺ T cells were isolated (>95% purity) using the EasySep PE positive selection kit (STEM-CELL Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions. Total RNA was collected using the miRNeasy minikit (Qiagen, Valencia, CA), and quality of RNA was confirmed spectrophotometrically. RNA integrity was validated using the Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). Next, we performed expression profiling of miRNAs using the

Affymetrix GeneChip miRNA 1.0 array platform (Affymetrix, Santa Clara, CA). The array included 609 murine-specific probes from Sanger miRBase (v11). A heat map was generated using a normalized log 2 expression value, i.e., normalized expression = $[\log_2 \text{Exp} - \text{average}(\log_2 \text{Exp})] / \text{average}(\log_2 \text{Exp}) * 100$, and hierarchical clustering was performed. To validate miRNA expression, the miScript cDNA synthesis kit (Qiagen) was used followed by quantitative real-time polymerase chain reaction (qRT-PCR) using the miScript SYBR Green PCR kit (Qiagen). Fold change of miRNAs was determined using the $2^{-\Delta\Delta C_t}$ method and expressed relative to Snord96a. For target mRNA validation, the SSO advanced SYBR Green PCR kit (Bio-Rad, Hercules, CA) was used to carry out qRT-PCR. Fold change was expressed relative to β -actin. The primers used in the study are highlighted in Table 1.

Transfection with miR-16 Mimic. CD4⁺ T cells were purified by magnetic bead separation as described earlier and cultured in complete (10% fetal bovine serum, 10 mM L-glutamine, 10 mM Hepes, 50 μ M β -mercaptoethanol, and 100 μ g/ml penicillin) RPMI 1640 medium (Gibco Laboratories, Grand Island, NY). Cells were seeded at 2×10^5 cells/well in a 24-well plate and transfected for 24 hours with mock control or 40 nM synthetic mmu-miR-16-6p (MSY0000527) using HiPerFect transfection reagent (Qiagen), according to the manufacturer's instructions. Total RNA and protein were extracted for analysis.

Western Blotting. Protein extracts (~15 μ g) were separated on 12% PAGE and transferred to a nitrocellulose membrane. The membranes were probed against B-cell lymphoma-2 or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Dallas, TX) and cyclin E1 (Cell Signaling Technology, Danvers, MA). Blots were developed using WesternSure PREMIUM Chemiluminescent Substrate and scanned using the C-Digit Blot Scanner from Licor (Lincoln, NE).

Bioinformatics Analysis. A total of 609 miRNAs were analyzed and sorted using the commercially available analysis tool Ingenuity Systems Ingenuity Pathway Analysis (IPA) software analysis (Mountain View, CA). In addition, in silico analysis of miRNA target genes was conducted using the Ingenuity knowledge base that combines data from miRBase (<http://www.mirbase.org/>), TarBase (<http://diana.cslab.ece.ntua.gr/tarbase/>), and Target Scan Human (<http://www.targetscan.org/>) target prediction softwares. Gene ontology enrichment analysis was performed using Cytoscape (<http://www.cytoscape.org/>), an open-source bioinformatics software. GENE-E (<http://www.broadinstitute.org/cancer/software/GENE-E/>) was used to generate the heat map of only those miRNAs that were overexpressed over 2.5-fold.

[³H]Thymidine Proliferation Assay. Primary T cells were isolated from lymph nodes from 2D2 transgenic mice, and bone marrow-derived dendritic cells (BMDCs) were generated as described

previously (Lutz et al., 1999). T cells and BMDCs were cocultured at the indicated ratios in the presence of 30 μ g/ml MOG₃₅₋₅₅ for 3 days. Cocultured cells were treated with DIM at 50 or 100 μ M. [³H]Thymidine was added to cultures 16 hours prior to harvesting.

Cell Cycle Analysis. Primary T cells were isolated from naïve C57BL/6 mice and cultured as naïve or activated with ConA (2.5 μ g/ml) in the absence or presence of vehicle or DIM (100 μ M) for 24 hours. Cells were harvested and fixed with 2% paraformaldehyde for 1 hour at 4°C. After washing with staining buffer, cells were incubated in a solution containing 0.2% Tween 20, 100 U/ml RNase, and 50 μ g/ml propidium iodide for 20 minutes at 37°C. Cell cycle analysis was performed using flow cytometry in which samples were gated on live cells.

Effects of DIM on Apoptosis of Naïve and ConA-Activated T Cells. Primary T cells were isolated from naïve C57BL/6 mice and cultured as naïve or activated with ConA (2.5 μ g/ml) in the absence or presence of DIM (100 μ M) or caspase 3 inhibitor. Inhibitors were added 2 hours prior to the administration of treatment as indicated. After 48 hours, cells were harvested and analyzed for terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling–positive staining via flow cytometry in which samples were gated on all cells.

Statistics. The data shown in this paper represent at least three independent experiments. The mean $\pm$ S.E.M. is shown for experiments that are applicable. The statistical differences within experiments were calculated using analysis of variance, whereas differences between groups were analyzed with the Bonferroni post-hoc test. Clinical scores were evaluated using groups of at least five mice using the Mann–Whitney test. Clinical studies were repeated at least three times with consistent results. A *P* value <0.05 was considered to be statistically significant.

Results

DIM Mitigates the Development of EAE. In the current study, we sought to investigate if the natural plant-derived indole DIM could serve as a novel therapeutic agent in mice induced with EAE, a murine model of the autoimmune disease MS. Ten days following immunization with MOG₃₅₋₅₅ peptide, when early signs of clinical disease were observed, EAE-induced mice were treated with vehicle (DMSO + corn oil) or DIM i.p. daily until the end of the study at day 25. Previous studies from our laboratory have shown that 40 mg/kg DIM was the lowest effective dose to significantly delay the onset of disease post-MOG immunization (Rouse et al., 2013). As evidenced by the clinical scores, indole-treated EAE mice exhibited a marked reduction in the severity of clinical symptoms and improved motor function compared with vehicle-treated EAE mice (Fig. 1A). In addition, DIM treatment lowered the incidence of EAE (Fig. 1B). Among the mononuclear cells isolated from brain, EAE mice treated with vehicle contained a significant number of CD3⁺, CD4⁺, and CD8⁺ T cells compared with naïve mice (Fig. 1C). Post-treatment with DIM considerably reduced the absolute number of infiltrating T cells. Upon examination of spinal cord histopathology, DIM post-treatment reduced the degree of cellular infiltration and tissue damage compared with EAE + vehicle mice (Fig. 1D).

DIM Treatment Modulates T-Cell Responses upon Activation. To evaluate the anti-inflammatory properties of DIM in a direct and antigen-dependent manner, we first isolated splenocytes from naïve and EAE + vehicle mice at the peak of the disease, day 15. Subsequently, cells were cultured for 3 days in the presence of MOG₃₅₋₅₅ along with vehicle or varying doses of DIM based on the bioavailability and calculated human equivalent dose (Stresser et al., 1995; Anderton et al., 2004).

TABLE 1
Real-time primers (RT-PCR) used

Primers	Primer Ssequence (5' → 3')
<i>Bcl2</i>	
Bcl2-F	ACT GAG TAC CTG AAC CGG CA
Bcl2-R	ACT TGT GGG CCA GGT ATG C
<i>Cyclin E1</i>	
CyclinE1-F	GTG GCT CCG ACC TTT CAG TC
CyclinE1-R	CAC AGT CTT GTC AAT CTT GGC
<i>Cyclin D1</i>	
CyclinD1-F	GTG GCT CCG ACC TTT CAG TC
CyclinD1-R	CAC AGT CTT GTC AAT CTT GGC
<i>ErbB3</i>	
ErbB3-F	TCT GCA TTA AAG TCA TCG AGG AC
ErbB3-R	CAG CCG TAC AAT GTG GGC AT
<i>ErbB4</i>	
ErbB4-F	GTG CTA TGG ACC CTA CGT TAG T
ErbB4-R	TCA TTG AAG TTC ATG CAG GCA A
<i>Runx1</i>	
Runx1-F	GCA GGC AAC GAT GAA AAC TAC T
Runx1-R	GCA ACT TGT GGC GGA TTT GTA

Fig. 1. DIM mitigates the development of EAE. (A) Clinical scores of EAE mice post-treated with vehicle ($n = 15$) or DIM ($n = 15$) (* $P < 0.05$ compared with EAE + vehicle). (B) Incidence of EAE development. (C) Mononuclear cells were isolated from brain on day 15 from EAE mice given vehicle ($n = 5$) or DIM ($n = 5$). The cells were stained with monoclonal antibody to identify CD3⁺, CD4⁺, and CD8⁺ T cells. Calculating the percentage of T-cell subsets determined by flow cytometry multiplied by absolute number of cells found in the brain and divided by 100 gave the absolute cell number for CD3⁺, CD4⁺, and CD8⁺ T cells, which were then plotted (* $P < 0.05$ compared with EAE + vehicle). (D) Representative images of spinal cord histopathology stained with H&E (10 $\times$) at day 15. Architecture is noted as white matter (WM) and gray matter (GM). Data are representative of three independent experiments.

MOG₃₅₋₅₅-stimulated splenocytes from EAE + vehicle mice were found to secrete a significantly higher amount of IFN γ in the in vitro supernatants compared with naïve mice (Fig. 2A). DIM treatment, however, led to a dose-dependent decrease in IFN γ secretion and was nondetectable at 100 μ M.

Next, we assessed the effects of DIM on T-cell activation. Upon culturing primary T cells with ConA, a potent T-cell mitogen, T cells displayed substantial increases in early activation markers CD69 and CD25 as well as costimulatory molecule CD28 within 24 hours compared with naïve T cells (Fig. 2B). When primary T cells were cultured in the combination of ConA and DIM, indole treatment significantly suppressed the expression of these critical markers, thus impeding ConA-induced activation.

The previous experiments established that DIM treatment was protective during the development of EAE by hindering T-cell responses. To further determine the extent of DIM treatment on T-cell activation and proliferation, we cocultured mitomycin C-treated BMDCs generated from wild-type mice and purified MOG-specific T cells from 2D2 transgenic mice at two different ratios in the presence of MOG₃₅₋₅₅ peptide. Using [³H]thymidine incorporation assay, we observed that the combination of BMDCs and 2D2 T cells resulted in significant T-cell proliferation compared with when each cell type was cultured alone with MOG₃₅₋₅₅ (Fig. 2C). In the presence of DIM, antigen-specific T-cell proliferation was found to dramatically decrease in a dose-dependent manner.

Exposure to DIM Leads to G0/G1 Arrest and Apoptosis in ConA-Activated T Cells. After observing DIM's effect on T-cell activation and proliferation, we wanted to determine the impact of DIM on cell cycle and apoptotic

processes in T cells. Therefore, we cultured primary T cells as naïve or activated with ConA. Following 24-hour treatment with vehicle or DIM, T cells were analyzed for cell cycle using propidium iodide staining and flow cytometry (Fig. 3A). As expected, the majority of the naïve T cells cultured with vehicle were found to be in G0/G1 phase with a relatively low percentage of cells in S and G2/M phases. When naïve T cells were cultured in the presence of DIM (50 μ M), we noted similar proportions of cells in most phases of cell cycle. ConA-activated T cells, on the other hand, were primarily in S phase after 1 day of culturing in comparison with naïve T cells. Interestingly, in the presence of DIM, almost half of the ConA-activated cells remained in G0/G1, whereas the other half were able to continue into S phase. These results suggest DIM impedes cell cycle progression in activated, but not naïve, T cells.

Arrest in G0/G1 as seen at 24 hours can trigger apoptosis in such cells. Thus, we analyzed the ConA-activated T cells for apoptosis at a later time point following culture with vehicle or DIM (Fig. 3B). After 48 hours, DIM-treated cells showed a significant increase in apoptosis in ConA-activated T cells compared with vehicle, from $4.2 \pm 0.2\%$ to $84.9 \pm 0.5\%$, respectively. Meanwhile, it is important to note that DIM treatment after 48 hours did not induce further apoptosis in naïve T cells ($33.4 \pm 2.2\%$ versus $35.4 \pm 4.4\%$). Upon the addition of caspase 3 inhibitor in the culturing conditions with DIM, the degree of apoptosis was significantly decreased ($84.9 \pm 0.5\%$ to $58.9 \pm 1.1\%$, respectively), suggesting indole-induced apoptosis may be mediated through activation of the caspase signaling pathway. Collectively, these results confirm DIM's ability to

Fig. 2. DIM treatment modulates T-cell responses upon activation. (A) Splenocytes from naive ($n = 5$) and EAE mice ($n = 5$) were isolated 15 days after disease induction. Cells were restimulated with MOG₃₅₋₅₅ peptide (30 μ g/ml) and treated with either vehicle or the indicated doses of DIM for 3 days. Supernatants were collected and analyzed for IFN γ . Data are expressed as the mean $\pm$ S.E.M. and statistical significance is indicated as ** $P < 0.01$ and *** $P < 0.001$ compared with EAE + vehicle. (B) Primary T cells were isolated and cultured as naive or activated with ConA (2.5 μ g/ml). Cells were simultaneously cultured in the presence or absence of vehicle or DIM (100 μ M) for 24 hours. The cells were analyzed using a flow cytometer and expressed as percent positive. Data are expressed as the mean $\pm$ S.E.M. and statistical significance is indicated as * $P < 0.05$ compared with ConA. (C) T cells purified from 2D2 mice ($n = 5$) were cocultured with BMDCs from C57BL/6 mice ($n = 3$) in the presence of MOG (30 μ g/ml) and vehicle or DIM for 72 hours. Cultures were pulsed with [3 H]thymidine 14 hours prior to harvesting. Data are presented as the mean $\pm$ S.E.M. of triplicate culture and statistical significance is indicated as * $P < 0.05$ compared with control. Data are representative of three independent experiments. CO, coculture; DC, dendritic cell; N.D., not detectable; WT, wild type.

regulate cell cycle and apoptotic processes in activated T cells.

Differentially Expressed miRNAs in Brain CD4⁺ T Cells upon DIM Treatment in EAE Mice. Recently, there have been a number of reports implicating miRNAs in the regulation of T cell-mediated inflammation and disease progression (O'Connell et al., 2010a; Tian et al., 2012; Sethi et al., 2013). Therefore, we investigated if DIM's effects on T-cell responses were due to altered miRNA expression, specifically in CD4⁺ T cells that had infiltrated the brain during disease progression. At the peak of the disease (day 15), we isolated CD4⁺ T cells from brain tissue of EAE mice post-treated with vehicle or DIM. The total RNA from these samples was analyzed using Affymetrix GeneChip miRNA 1.0 array platform. The heat map of the miRNA data illustrated that DIM post-treatment in EAE mice led to significant changes in the miRNA profile compared with vehicle-treated EAE mice (Fig. 4A). Among the 609 miRNAs analyzed, we found 53 miRNAs were upregulated by more than 2-fold when treated with DIM, whereas 11 miRNAs were downregulated (Fig. 4, B

and C). After conducting the miRNA microarray, Ingenuity Systems IPA knowledge base software highlighted a list of "top upregulated" miRNAs that were induced upon DIM post-treatment during EAE. These miRNAs, which comprised miR-200c, miR-93, miR-16, miR-22, and miR-146a, are highlighted along with their mature full-length sequence, associated seed sequence, and respective fold changes (Table 2). Subsequently, we validated these five miRNAs and found that post-treatment with DIM significantly led to their upregulation in brain CD4⁺ T cells of EAE mice (Fig. 4D), suggesting their potential role in mediating the observed antiproliferative and proapoptotic role of DIM treatment during the progression of EAE.

Predicted Targets of Differentially Expressed miRNAs in Brain CD4⁺ T Cells upon DIM Treatment in EAE Mice. IPA analysis revealed that a number miRNAs, particularly the five select miRNAs that we validated, were associated with critical biological functions and pathways, such as cell signaling, regulation of gene expression, proliferation, development, and survival (Fig. 5A). Additionally, Cytoscape,

Fig. 3. Exposure to DIM leads to G0/G1 arrest and apoptosis in ConA-activated T cells. T cells from C57BL/6 mice ($n = 5$) were cultured as naive or activated with ConA (2.5 $\mu\text{g/ml}$). (A) Cells were cultured in the presence of either vehicle or DIM (100 μM) for 24 hours and analyzed for cell cycle progression using flow cytometry (gated on live cells). (B) Naive and ConA-activated T cells were cultured in the presence of either vehicle or DIM (100 μM) for 48 hours. Caspase 3 inhibitor was added at a final concentration of 20 μM , and samples were analyzed for apoptosis based on terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling-positive staining (gated on all cells). Data are representative of three independent experiments.

a tool that assigns specific gene ontology-based functions, was used to gain further insight into the biological roles of these particular miRNAs. Interestingly, we found that the target genes of the top upregulated miRNAs were primarily involved in cell cycle and apoptosis (Fig. 5B). These results strongly support a role for DIM-induced miRNAs in the halting of cell cycle progression and induction of apoptosis that we observed earlier in our study (Fig. 3, A and B).

To validate the target genes of the DIM-induced miRNA, we first filtered the highly predicted target genes based on their role in promoting cell cycle progression and cellular proliferation using IPA. Further, we focused on only those genes that were experimentally observed to be targeted by DIM-induced miRNA (Table 3). Since the targeting and subsequent degradation of miRNA target genes is dependent on the appropriate binding of the miRNA seed sequence to the 3'-untranslated region (3'-UTR) of the respective target genes, we schematically depicted the putative binding sites for the validated miRNAs on the 3'-UTR of various cell cycle and apoptotic mRNA target genes (Fig. 6A). To determine if DIM post-treatment could regulate the expression of some of these experimentally confirmed miRNA target genes within the disease model, we performed qRT-PCR on CD4⁺ T cells that were isolated from the brains of EAE + vehicle or EAE + DIM mice at the peak of disease. In brain-infiltrating CD4⁺ T cells, we found that DIM post-treatment was able to significantly reduce the expression of *Bcl2*, *Cyclin D1* (*Ccnd1*), *Cyclin E1* (*Ccne1*), *V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 3* (*ErbB3*) and *V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 4* (*ErbB4*), and *runt-related transcription factor 1* (*Runx1*), all of which contribute to driving cell growth and survival (Fig. 6B).

To further confirm the role of DIM-induced miRNAs on target genes associated with cell cycle and apoptotic processes, we transfected CD4⁺ T cells with miR-16 mimic.

Twenty-four hours after the transfection, we determined that miR-16 expression increased 25-fold compared with mock control (Fig. 6C). Moreover, we found that the overexpression of miR-16 led to the significant reduction in target genes *Ccne1* and *Bcl2*, thus confirming the influential role of DIM-induced miRNAs on regulating cell cycle progression and apoptosis. Taken together, the inverse relationship between DIM-induced miRNAs and their respective target genes suggests that DIM mediates its protective effects, at least in part, due to its ability to modulate miRNAs involved in cell cycle progression and apoptosis.

Interaction Network of DIM-Induced Validated miRNAs and Respective Target Genes. In the current study, we set out to investigate the beneficial effects of DIM on the development of EAE and determined that DIM was able to modulate key miRNAs in CD4⁺ T cells that infiltrated the brain during EAE. Based on our findings, the predicted and experimentally supported target genes of differentially expressed DIM-induced miRNA in brain CD4⁺ T cells were combined and analyzed for their significant association with canonical pathways (Fig. 7). Using an IPA-generated network, we highlighted the intricate net of cell cycle and apoptotic signaling pathways impacted by DIM-induced miRNA. Additionally, we found that a number of these miRNAs converge upon common target genes, suggesting that DIM-induced miRNAs may act independently or collectively to orchestrate the regulation of cell cycle progression and apoptosis in activated T cells during EAE.

Discussion

Multiple sclerosis is a debilitating neurodegenerative disease characterized by the generation of inflammatory cytokines and autoreactive immune cells. Currently, there is no cure for this disease, and treatment is restricted to the use of

Fig. 4. Differentially expressed miRNAs in brain CD4⁺ T cells upon DIM treatment in EAE mice. Total RNA was isolated and pooled from CD4⁺ T cells obtained from the brains of EAE mice post-treated with vehicle (*n* = 5) or DIM (*n* = 5) on day 15. MicroRNA microarray expression levels of differentially expressed miRNAs were generated by deep sequencing. (A) Unsupervised hierarchical clustering of the relative expression of 609 miRNAs. The color scale denotes those miRNAs that were upregulated (red) and downregulated (green). (B) The fold change distribution of the 609 miRNAs found within CD4⁺ T cells from the brains of EAE + vehicle and EAE + DIM mice. (C) Proportional Venn diagram illustrating fold change of miRNAs that were 2-fold dysregulated following post-treatment with DIM in EAE mice. (D) Expression levels of selected upregulated miRNAs were analyzed using Snord96a as a small RNA endogenous control. Data are presented as the mean $\pm$ S.E.M. and statistical significance is indicated as **P* < 0.05; ***P* < 0.01 compared with EAE + vehicle. Data are representative of three independent experiments.

immunosuppressive drugs, such as mitoxantrone, cladribine, and teriflunomide (Fox, 2006; Giovannoni et al., 2010), or therapeutic agents that inhibit cell migration, such as natalizumab and fingolimod (Chiba and Adachi, 2012; Rinaldi et al., 2012). Alternatively, immunomodulatory strategies to alter the course of MS have been used, such as daclizumab, an anti-CD25 monoclonal antibody that promotes the induction of regulatory immune cells leading to the inhibition of activated T cells (Bielekova et al., 2009), as well as alemtuzumab or rituximab, which have been used to target specific cell surface markers, such as CD52 or CD20, or to deplete T and B cells, respectively (Bates, 2009; Hawker et al., 2009; Coles, 2013).

However, the majority of these treatment options only appear to slow the progression of disease, while carrying significant risk factors and side effects. Long-term use of immunosuppressant drugs, for example, can lead to increased risk of infection or secondary diseases (Fox, 2006; Confavreux et al., 2012; Coles, 2013). Also, β interferons and drugs such as fingolimod and teriflunomide have been reported to cause toxicity to vital organs, such as the liver and heart (Walther and Hohlfeld, 1999). Due to the toxic side effects of some

treatments, there still remains the need for safer and more effective treatment options for patients with MS. In the current study, we demonstrate that DIM, a natural plant-derived compound, alleviates the clinical signs of MS through immunomodulatory mechanisms that include the induction of critical miRNAs involved in regulating apoptosis and cell cycle progression. In addition, the dosages used have been noted to be safe and nontoxic (Stresser et al., 1995; Anderton et al., 2004).

Previous studies have also demonstrated the beneficial effects of DIM. For example, we have demonstrated in our previous work that DIM can modulate T cells by decreasing Th17 cells while promoting the induction of T regulatory cells (Rouse et al., 2013). Another report showed that DIM reduces inflammatory cytokines as well as the expression of nuclear factor- κ B ligand, thus attenuating the development of arthritis (Dong et al., 2010). Additionally, DIM was concluded to be anti-inflammatory upon effectively inhibiting 12-*O*-tetradecanoylphorbol-13-acetate-induced ear edema by downregulating cyclooxygenase-2, interleukin-6, and nuclear factor- κ B (Kim et al., 2010). In the current study, we investigated the therapeutic potential of DIM for the treatment of MS. Our

TABLE 2

Highly upregulated miRNAs upon DIM post-treatment in EAE mice

To generate a list of top upregulated miRNAs, 609 miRNAs were analyzed using IPA knowledge base, which is composed of miRBase (<http://www.mirbase.org/>), TarBase (<http://diana.cslab.ece.ntua.gr/tarbase/>), Target Scan Human (<http://www.targetscan.org/>), and Ingenuity expert findings. The arrows indicate the fold change expression as determined by microarray miRNA deep sequencing.

MicroRNA Identification	MicroRNA Sequence	Seed Sequence	Fold Change
mmu-miR-200c	UAAUACUGCCGGUAAUGAUGGA	AAUACUG	5.8 ↑
mmu-miR-93	CAAAGUGCUUACAGUGCAGGUAG	AAAGUGC	5.7 ↑
mmu-miR-16	UAGCAGCAGUAAAUAUUGGC	AGCAGCA	3.7 ↑
mmu-miR-22	AAGCUGCCAGUUGAAGAACUGU	AGCUGCC	5.1 ↑
mmu-miR-146a	UGAGAACUGAAUUCGAUGGGUU	GAGAACU	4.8 ↑

findings demonstrate that DIM could significantly reduce the severity of clinical scores of EAE mice even when administered 10 days after the disease induction. Furthermore, we show that treatment with DIM post-treatment strongly reduces cellular infiltration, a hallmark for EAE, and decreases CNS histopathological changes commonly associated with the disease.

While several studies have demonstrated that DIM provides beneficial effects in diseases such as cancer, the underlying molecular mechanisms behind the protective effects of DIM in other disease models have not been well established. Recently, a number of reports have highlighted the role of miRNAs in driving immunomodulatory processes. miRNAs are noncoding RNA molecules that interact with 3'-UTR regions of mRNA targets with complimentary binding sites.

As a consequence, a number of miRNAs can target hundreds of genes in concert, leading to significant changes in overall gene expression (He and Hannon, 2004). In the current study, we hypothesized that DIM treatment may regulate critical miRNAs, which could potentially shed light on the beneficial health effects associated with DIM. We demonstrate that DIM could significantly diminish peripheral T-cell activation and function, thus hindering the progression of EAE. Furthermore, using a combinatorial miRNA analysis approach, we found that DIM post-treatment can still elicit striking global effects by dysregulating a number of miRNAs in CD4⁺ T cells that had infiltrated the brains of EAE mice. In particular, we observed a pronounced expression of five miRNAs: miR-200c, miR-93, miR-16, miR-22, and miR-146a. Upon

Fig. 5. Functional analysis of differentially expressed miRNAs in brain CD4⁺ T cells upon DIM treatment in EAE mice. (A) Bar chart of significant ($P < 0.05$) biological functions associated with highly upregulated miRNAs as determined by IPA. The y-axis represents the functional categories and the x-axis the significance score (negative log of P value) as determined by Fisher's exact test. (B) Gene ontology network highlighting critical biological functions of the predicted target genes common to miR-200c, miR-146a, miR-22, miR-93, and miR-16. The network was generated by Cytoscape 3.0 using ClueGo v2.0.6 + CluePedia v1.06 application. κ score threshold was set at 0.3 and statistical significance was determined by a two-sided hypergeometric test with Benjamini Hochberg correction. JAK, Janus kinase; STAT, signal transducer and activator of transcription.

TABLE 3

Experimentally observed targets of highly upregulated miRNAs pertinent to cell proliferation, cell cycle progression, and apoptosis

Ingenuity Pathway Analysis was used to generate a list of experimentally observed targets of the validated miRNAs filtered on cell proliferation, cell cycle progression, and apoptosis criteria.

miRNA	Experimentally Observed Targets
miR-200c	PLCG1, ZEB1, ZEB2, CDKN1B, BCL2
miR-93	CCND1, CDKN1A, CREB1, E2F1, S1PR1, PAK7, S1PR1, TGFBR2, VIM, RUNX1, RB1, BCL2
miR-16	BCL2, CCND1, CCNE1, CDK6, CLDN12, HSP90B1, ITGA2, MAP2K1, MAP2K4
miR-22	ERBB3, ESR1
miR-146a	FADD, NFKB1, STAT1, CXC3R4, IRAK1, ERBB4

CDK6, cyclin-dependent kinase 6; CDKN1B, cyclin-dependent kinase inhibitor 1B; CLDN12, claudin 12; CREB1, cAMP response element-binding protein 1; CXC3R4, c-x-c motif 3R4; E2F1, E2F transcription factor 1; ESR1, estrogen receptor 1; FADD, Fas-associated protein with death domain; HSP90B1, heat shock protein 90B1; IRAK1, interleukin-1 receptor-associated kinase 1; ITGA2, integrin α 2; MAP2K1, mitogen-activated protein kinase kinase 1; MAP2K4, mitogen-activated protein kinase kinase 4; NFKB1, nuclear factor- κ B; PLCG1, phospholipase C γ 1; RB1, retinoblastoma protein; S1PR1, sphingosine 1 phosphatase receptor 1; STAT1, signal transducers and activators of transcription 1; TGFBR2, transforming growth factor β receptor 2; VIM, vimentin; ZEB, zinc finger e-box binding homeobox.

using a bioinformatics approach to study the functional significance of these miRNAs, we noted that these miRNAs were collectively involved in processes such as cellular proliferation, cell cycle progression, and cell death and survival. These findings indicated that the extent of DIM's beneficial properties include local as well as systemic effects on immune cell responses.

Previous studies have explored the role of these particular miRNAs in cancer. For example, the overexpression of miR-200b/c sensitizes several multidrug-resistant gastric and lung cancer cell lines to vincristine- and cisplatin-induced apoptosis (Zhu et al., 2012). The study verified that miR-200c targets *Bcl2*, an antiapoptotic molecule, to trigger apoptosis. Additionally, a report conducted in chronic lymphocytic leukemia demonstrated an inverse relationship between miR-16 and *Bcl2* (Cimmino et al., 2005). Similarly, miR-15 and miR-16 were downregulated in patients with relapsing-remitting multiple sclerosis, which corresponded with the increase in *Bcl2* in CD4⁺ T cells (Lorenzi et al., 2012). As a result, increased *Bcl2* levels protected autoreactive T cells from undergoing apoptosis. In the current study, we validated the expression of miR-200c and miR-16 through qRT-PCR. Further, we not only confirmed the downregulation of *Bcl2* in CD4⁺ T cells isolated from brains of EAE mice post-treated with DIM, but also found that overexpression of miR-16 with a synthetic mimic could repress *Bcl2* expression. Therefore, taken together, our data demonstrate that the observed induction of cell cycle arrest and apoptosis in activated T cells by DIM can be explained by the induction of particular miRNAs that target cell-proliferating and antiapoptotic genes.

Several miRNAs share common “seed sequences,” 6–8 nucleotides at their 5' end that determine mRNA target specificities. Therefore, miRNA families that comprise common seed sequences are likely to share common targets. For example, the miR-16 family, which comprises miR-15a, miR-16, and miR-195, targets *Ccnd1*, *Ccne1*, and *Bcl2*, thereby inhibiting cell cycle progression and promoting apoptosis (Liu et al., 2008). Similarly, the miR-17/20/106 family, which consists of miR-17, miR-106a, miR-20a, miR-20b, and miR-93, regulates gene targets involved in G₁/S transition (Kumar et al., 2013). The miR-17/20 family also negatively regulates CCND1 in breast cancer cells (Yu et al., 2008). In the current study, we observe the repression of cell cycle-specific genes

Ccnd1 and *Ccne1* upon DIM post-treatment and the corresponding increase in expression of miR-93 and miR-16 that regulates their expression. Furthermore, overexpression of miR-16 using a synthetic mimic also led to the significant downregulation of *Ccne1* in CD4⁺ T cells. In addition to suppressing these genes, we observed that DIM treatment in vitro was able to induce G₀/G₁ arrest in ConA-activated T cells at 24 hours without impeding cell cycle progression in naïve T cells. These data highlight the potential role of DIM-induced miRNAs in restricting cell cycle progression in activated T cells.

Runx1, also known as acute myeloid leukemia-1, is significantly upregulated in several types of leukemia (Asou, 2003; Gattenloehner et al., 2007) as well as in breast cancer (Cancer Genome Atlas Network, 2012). Additionally, *Runx1* upregulation is associated with progression of G₁/S during cell cycle (Bernardin-Fried et al., 2004). Previous studies have reported that the miR-17/20/106 family has been shown to target *Runx1* (Fontana et al., 2007). In our study, we observed significant suppression of *Runx1* followed by increased expression of miR-93 upon DIM post-treatment during EAE. These findings suggest that DIM-mediated induction of miR-93, a member of the miR17-106 family, may once again play an important role in repressing cell cycle-associated genes.

A critical characteristic of EAE development is the inflammation and infiltration of autoreactive T cells. Studies have shown miR-146a can act as a critical regulator in such processes. For example, miR-146-null mice have been reported to possess enhanced immune cell activation and levels of inflammation, leading to the pronounced development of autoimmune disease and tumors (Boldin et al., 2011). In our study, we found that EAE mice treated with DIM exhibit an increased expression of miR-146a in brain-infiltrating CD4⁺ T cells, whereas expression of its target *Erbb4*, a receptor tyrosine kinase that promotes proliferation of cells (Horie et al., 2010), was significantly reduced. Upon examining the effects of DIM on T-cell clonal expansion in response to MOG antigen, we observed that DIM treatment significantly inhibits antigen-specific proliferation. Furthermore, we find that indole treatment limits T-cell activation as confirmed by the reduced expression of CD69, CD25, and CD28 in ConA-activated T cells, thus suggesting antiproliferative and anti-inflammatory roles for DIM.

Fig. 6. Experimental validation of in silico-predicted miRNA targets. Total RNA was obtained from pooled CD4⁺ T cells that were isolated from the brains of EAE + vehicle ($n = 5$) or EAE + DIM mice ($n = 5$) on day 15. (A) miRanda (Computational Biology Center of Memorial Sloan-Kettering Cancer Center, New York, NY)-generated alignments of target binding sites on 3'-UTR of selected target genes involved in cell cycle and apoptotic processes. (B) Expression levels of selected target genes of top upregulated miRNAs were analyzed using β -actin as an endogenous control. Data are presented as the mean $\pm$ S.E.M. from triplicate samples (* $P < 0.05$; ** $P < 0.01$ compared with EAE + vehicle). (C) Purified CD4⁺ T cells from naive C57BL/6 mice were transfected with mock control or synthetic mmu-miR-16-6p. Total RNA was extracted 24 hours later to assess relative expression of miR-16, *Ccne1*, and *Bcl2* by qRT-PCR. Expression levels of miR-16 were analyzed using Snord96a as a small RNA endogenous control. Expression levels of *Ccne1* and *Bcl2* were analyzed using β -actin as an endogenous control. (D) Purified CD4⁺ T cells from naive C57BL/6 mice were transfected with mock control or synthetic mmu-miR-16-6p. Protein was extracted 24 hours later and western blot analysis was performed to assess expression levels of miRNA targets *Ccne1* and *Bcl2*, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Data are presented as the mean $\pm$ S.E.M. from triplicate samples (** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared with mock control).

Previously DIM has been reported to effectively control cellular growth and viability through the induction of cell cycle arrest and/or apoptosis. However, these studies have predominantly involved the development of cancer (Lerner et al., 2012; Li et al., 2013). Similarly, studies have shown that miR-22 can protect against cancer as illustrated by the fact that overexpression of miR-22 is associated with reduced tumor growth, volume, and decreased migration of tumor cells (Ling et al., 2012). Part of miR-22's protective effects are due to its ability to bind the 3'-UTR of *ErbB3*, a gene associated with the growth and progression of various cancers (Kraus et al., 1989; Lee and Maible, 1998). Our findings highlight that DIM and miR-22 can also prompt similar therapeutic

effects upon activated immune cells, particularly T cells. In the current study, we report that DIM post-treatment enhanced the expression of miR-22 in brain CD4⁺ T cells, subsequently leading to the reciprocal reduction in the expression of *ErbB3*.

In conclusion, the current study demonstrates the therapeutic potential of DIM in ameliorating the development of the autoimmune disease EAE. We observe that the immunomodulatory and anti-inflammatory activity of DIM is a result of its direct effect on activated T cells. In particular, our study is the first to show that the ability of DIM to induce cell cycle arrest and apoptosis in activated T cells is linked closely with the induction of key miRNAs that act collectively to restrict

Fig. 7. Interaction network of validated miRNAs and their confirmed target genes. IPA was used to generate an interaction network of validated DIM-induced miRNAs and their respective target genes. The symbols as generated by IPA are represented as follows: miRNA (red), transcription factors (blue), kinases (pink), transporter (yellow), and other (orange). NFAT5, nuclear factor of activated T cells 5.

multiple genes that would otherwise contribute to the development and progression of EAE.

The current study has identified key microRNAs that potentially regulate the T-cell proliferation and apoptosis induced by DIM. Further studies aimed at the modulation of these miRNA in vivo may shed light on their precise role in EAE. Based on the ability of DIM-induced miRNA to influence multiple genes, it is important to continue exploring additional DIM-induced miRNAs and their respective target genes as potential therapeutic targets. Additionally, since DIM is commonly used as a dietary supplement, it would also be interesting to assess the effects of DIM on miRNA that are dysregulated in MS patients to build on our knowledge of DIM's mechanisms of action and role in translational medicine. Although the mechanism by which DIM induces miR-200c, miR-146a, miR-16, miR-93, and miR-22 remains unknown, our study notably demonstrates the beneficial impact of DIM on modulating activated T cells during EAE through the induction of miRNAs that regulate cell cycle and apoptotic processes.

Authorship Contributions

Participated in research design: Rouse, Rao, M. Nagarkatti, P. S. Nagarkatti.

Conducted experiments: Rouse, Rao.

Contributed new reagents or analytic tools: Rouse, Rao, M. Nagarkatti, P. S. Nagarkatti.

Performed data analysis: Rouse, Rao, M. Nagarkatti, P. S. Nagarkatti.

Wrote or contributed to the writing of the manuscript: Rouse, Rao, M. Nagarkatti, P. S. Nagarkatti.

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