Immunopathology and Infectious Diseases

Immunomodulatory Effect of Combination Therapy with Lovastatin and 5-Aminoimidazole-4-Carboxamide-1-β-D-Ribofuranoside Alleviates Neurodegeneration in Experimental Autoimmune Encephalomyelitis

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Combination therapy with multiple sclerosis (MS) therapeutics is gaining momentum over monotherapy for improving MS. Lovastatin, an HMG-CoA reductase inhibitor (statin), was immunomodulatory in an experimental autoimmune encephalomyelitis (EAE) model of MS. Lovastatin biases the immune response from Th1 to a protective Th2 response in EAE by a different mechanism than 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, an immunomodulating agent that activates AMP-activated protein kinase. Here we tested these agents in combination in an EAE model of MS. Suboptimal doses of these drugs in combination were additive in efficacy against the induction of EAE; clinical symptoms were delayed and severity and duration of disease was reduced. In the central nervous system, the cellular infiltration and proinflammatory immune response was decreased while the anti-inflammatory immune response was increased. Combination treatment biased the class of elicited myelin basic protein antibodies from IgG2a to IgG1 and IgG2b, suggesting a shift from Th1 to Th2 response. In addition, combination therapy lessened inflammation-associated neurodegeneration in the central nervous system of EAE animals. These effects were absent in EAE animals treated with either drug alone at the same dose. Thus, our data suggest that agents with different mechanisms of action such as lovastatin and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, when used in combination, could improve therapy for central nervous system demyelinating diseases and provide a rationale for testing them in MS patients. (Am J Pathol 2006, 169:1012–1025; DOI: 10.2353/ajpath.2006.051309)

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS) that mimics many aspects of multiple sclerosis (MS).1 Pathophysiology of EAE includes breaching of the blood brain barrier, infiltration of mononuclear cells—predominantly myelin-reactive CD4+ and CD8+ T-cells and macrophages, resulting in the activation of resident CNS glial cells.2 Different from EAE, however, myelin-reactive CD8+ T cells play a major role in MS pathogenesis.3 On activation CNS glial cells secrete proinflammatory mediators [cytokines, chemokines, and inducible nitric oxide (NO) synthase] and activate complement cascade pathways.4 These events produce excitotoxic and oxidative damage because of the depletion of intracellular energy stores, destabilization of the cell membrane, opening of voltage-gated Ca2+ channels, and activation of NMDA receptors in neurons and oligodendrocytes.5–7 Moreover, depletion of ATP in astrocytes during EAE leads to mitochondrial malfunction and cellular energy failure, contributing to neuron loss.8,9 Consequently, these inflammatory events lead to CNS demyelination because of degradation of the myelin sheath and a loss of both oligodendrocytes and neuronal axons in the CNS.

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Various immunomodulatory agents with different mechanisms of action are being tested for MS treatment because presently approved therapies for MS are only partially effective and are associated with side effects and potential toxicities. Systemic administration of transforming growth factor (TGF)-β,10 the nucleoside analogue cladribine,11 and the leukocyte-depleting (CD52) monoclonal antibody campath-1H12 are all discouraged for use in MS treatment because of their adverse effects. Studies conducted with interferon (IFN)-β13 and glatiramer acetate14 were promising in some patients, but many individuals experienced poor responses or adverse effects.

Because of the inherent complexity of MS and the involvement of multiple cell types such as brain, endothelial, and vascular immune cells, evidence suggests that monotherapy with either pre-existing or new MS drugs will be insufficient for controlling the chronic progressive disability observed in affected individuals. One approach to improve treatment is to develop more efficacious agents and another, more plausible approach, is to identify possible combinations of existing or novel agents that together are additive/synergistic.15

Recently, cholesterol-lowering HMG-CoA reductase inhibitors (statins) have been exploited for their immunomodulatory characteristics for the treatment of MS patients.16–19 Promising results were obtained in initial clinical trials of simvastatin and atorvastatin in MS18 and rheumatoid arthritis,20 respectively. In animal studies, lovastatin17,19 and atorvastatin16 protected animals against both acute and remitting-relapsing EAE disease via attenuation of the neuroinflammatory CNS response and the promotion of Th2 differentiation of naïve myelin-specific T cells. Recently, we documented that lovastatin augments the remyelination process in the CNS of animals recovering from EAE via enhanced survival and differentiation of oligodendrocyte progenitors.21 Thus, one can envisage that an agent that augments immunomodulation of myelin-reactive T cells toward Th2 differentiation could be beneficial.

Recently, we also reported that AMP-activated protein kinase (AMPK) activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is a novel immunomodulator agent and a likely candidate for MS treatment.22 In animal models of endotoxemia23 and EAE,22 AICAR treatment protected against lipopolysaccharide-induced proinflammatory response in CNS glial cells as well as in remitting-relapsing EAE in SJL mice. Treatment of EAE mice with AICAR biased myelin-reactive T cells toward Th2 differentiation and was immunomodulatory in antigen-presenting cells via induction of anti-inflammatory cytokines.22 Under normal cellular conditions, a rise in AMP or an increase in the AMP/ATP ratio signals declining energy stores which in turn activate AMPK. This activation of AMPK can acutely regulate cellular metabolism and chronically regulate gene expression to restore ATP levels.24,25 Conversely, under CNS inflammation conditions observed in the MS brain, ATP depletion attributable to mitochondrial malfunction and glutamate accumulation increases neuronal apoptosis.7 In turn, the pharmacological activation of AMPK by AICAR increases the survival of hippocampal neurons under reduced energy conditions, ie, glucose deprivation and glutamate excitotoxicity,26 suggesting its neuroprotective role against CNS demyelination. Because lovastatin or AICAR treatment promotes the development of protective Th2 response using different mechanisms of action, we hypothesized that lovastatin and AICAR in combination could be a better approach to lessen inflammation-associated neurodegeneration in the CNS of EAE animals.

In this study we report that lovastatin and AICAR in combination are complementary in a synergistic or additive manner in EAE treatment. Combination therapy with suboptimal doses of lovastatin and AICAR (half of an individual optimal dose) additively reversed or prevented EAE in animals by reducing disease severity, CNS inflammation, and neurodegeneration, compared with animals treated with either drug alone at the same dose.

Materials and Methods

Reagents

Myelin basic protein (MBP) (−50% pure from guinea pig brain), complete Freund’s adjuvant (CFA), horseradish peroxidase-tagged anti-mouse IgG antibodies, and chemicals were purchased from Sigma (St. Louis, MO). Lovastatin was purchased from Calbiochem (San Diego, CA). AICAR was purchased from Toronto Research Chemicals (Toronto, ON, Canada). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA), and RNeasy cleaning kits were from Qiagen (Valencia, CA). Antibodies used include mouse anti-rat CD4 and CD8 and anti-mouse myelin basic protein (MBP) (clone 1, 129-138) from Serotec (Raleigh, NC). Rabbit anti-IFN-γ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-iNOS polyclonal antibodies were purchased from Upstate (Charlottesville, VA). Mouse anti-phosphorylated-neurofilament-heavy (SMI-31) and rabbit anti-myelin-associated glycoprotein (MAG) antibodies were purchased from Chemicon (Temecula, CA) and Zymed (Carlsbad, CA), respectively. Mouse anti-rat ED1 antibodies were purchased from Biosource (Camarillo, CA). Secondary antibodies include Texas Red-X-conjugated goat anti-mouse IgG (for MBP and SMI-31) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (for MAG), purchased from Vector Laboratories, Inc. (Burlingame, CA). Streptavidin Texas Red-conjugated (for ED1) and streptavidin fluorescein isothiocyanate-conjugated (for iNOS and IFN-γ) antibodies were supplied in TSA indirect kit purchased from Perkin-Elmer (Boston, MA).

Animals

Female Lewis rats (225 to 300 g) were purchased from Harlan Laboratory (Harlan, IN) and housed in the animal care facility of the Medical University of South Carolina throughout the experiment. Food and water were provided with ad libitum. All experiments were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication number 80-23, revised 1985) and were approved.
by the Medical University of South Carolina animal care and use committee.

**EAE Induction and Clinical Evaluation**

The procedures used for the induction of EAE have been described previously. In brief, female rats received a subcutaneous injection in the hind limb of MBP (50 µg) in 0.1 ml of phosphate-buffered saline (PBS) emulsified in an equal volume of CFA supplemented with 2 mg/ml of mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) on days 0 and 7. Immediately thereafter and again 24 hours later, rats received pertussis toxin (200 ng, i.p.) in 0.1 ml of PBS. Individual animals were observed daily, and clinical scores were assessed by an experimentally blinded investigator using a 0 to 5 scale: 0, no clinical disease; 1, piloerection; 2, loss in tail tonicity; 3, hind leg paralysis; 4, paraplegia; and 5, moribund or dead.

**Lovastatin and AICAR Treatments**

Lovastatin was suspended in 0.8% ethanol/0.6 N NaOH and PBS adjusted to pH 7.4. Likewise, AICAR was suspended in PBS as described previously. Lovastatin (1 or 2 mg/kg, i.p.) and AICAR (50 mg/kg, i.p.) were administered once daily using an insulin syringe (1 ml). Suboptimal doses of lovastatin (1 mg/kg) and AICAR (50 mg/kg) were chosen based on previous studies with lovastatin (2 mg/kg) and AICAR (100 mg/kg) and were used in combination or individually. For EAE prevention, rats received an injection of each drug (0.15 ml, i.p.) in combination or individually starting from day 0 of EAE induction. For EAE reversal, daily treatment with lovastatin and AICAR combination or either drug alone began when a clinical score of ≥2.0 was reached. Drug treatment was continued until the end of the experiment once it was started. EAE animals received an injection (intraperitoneally) of vehicle (placebo, 0.8% ethanol in PBS) once daily. Control animals received an injection (intraperitoneally) of either vehicle (placebo) or lovastatin and AICAR in combination once daily. Animals were sacrificed on peak clinical day, day 13 after immunization, or on remission (25 days after immunization) to collect serum and spinal cord (SC) tissues. Animals developing severe EAE disease after treatment with drug or placebo were sacrificed 14 days after immunization onwards as per animal protocol guidelines.

**Histopathology**

Because the most significant pathological changes in animals with EAE are detected in the lumbar region of the SC, for histopathological examinations, the lumbar region of the SC was fixed in 10% buffered formalin (Stephens Scientific, Riverdale, NJ) and embedded in paraffin to cut 4-µm-thick sections. Sections were stained with hematoxylin and eosin (H&E), luxol fast blue-hematoxylin (LFB), and Bielschowsky’s silver impregnation to assess inflammation, demyelination, and axonal pathology. Sections were mounted with aqueous mounting media (Vectorshield; Vector Laboratories) and examined under a light microscope (Olympus BX-60; Olympus, Tokyo, Japan), and images were captured with an Olympus digital video camera (Optronics, Goleta, CA) using a dual band pass filter using Adobe Photoshop 7 software. To quantify inflammation and demyelination in EAE, images of LFB-stained SC sections (n = 9) from each group were captured (∗×200), coded, and proceeded for counting of nuclei and LFB staining intensity in a blinded manner using Image-Pro Plus 4 image software (Media Cybernetics, Silver Spring, MD). Likewise, for quantification of axons, the total cross-sectional area of SC sections (n = 9) stained with Bielschowsky’s silver impregnation method from each group was measured from digital images captured at ×400. Axonal density was measured by manually counting on the converted image on grayscale using Image-Pro Plus 4. Total number of axons were calculated by multiplying the centrally samples density by the total cross-sectional area for the same SC section. These studies were performed in each group of animals in three identical experiments, computed, and plotted in Excel.

**Immunohistochemistry**

Immunostaining was performed on adjacent serial sections using standard techniques. For single labeling, sections were incubated with appropriately diluted primary antibody followed by washing and incubation with secondary antibodies (1:100) as described previously. Tyramide signal enhancement technique was used (Renaissance TSA for Immunocytochemistry; NEN Life Sciences, Boston, MA) per the manufacturer’s instructions for amplification of weak signals. For double-immunofluorescence labeling, sections were sequentially incubated with primary antibodies (dilution, 1:100), followed by washing and incubation again with matching secondary antibodies (dilution, 1:100). Sections were mounted and examined under fluorescence microscope (Olympus BX-60), and images were captured with an Olympus digital video camera using a dual-band pass filter. Manual counting of positive cells (CD4, CD8, and ED1) was performed on 10 fields per section from three animals per group in each experiment (n = 3). Immunofluorescence intensity in the white matter areas for MBP and MAG was quantified with Image-Pro Plus 4 as described above for quantification of demyelination. Intensities of SC sections (n = 9) were computed from three identical experiments. Data were plotted in Excel.

**RNA Extraction, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction (QPCR) Analysis**

Lumbar SC tissues were carefully processed for RNA isolation. RNA was purified using TRIzol reagent according to the manufacturer’s protocol as described previously. Single-stranded cDNA was synthesized from SC tissue RNA from each group of animals by using a superscript preamplification system for first-strand cDNA synthesis (Life Technologies Inc., Gaithersburg, MD) as described earlier. Real-time QPCR was performed using the iCycler IQ real-
time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The primer sets used were designed and purchased from integrated DNA technologies (IDT, Coralville, IA). The primer sequences were as follow: for GAPDH, forward primer (FP): 5’-CTTACACTTATCCGTTGAT-3’; reverse primer (RP): 5’-GGAGGAAGGAGGTGCTGTAAG-3’. IFN-γ: FP: 5’-ATTCCTCCCACCTACTTCAT-TAG-3’ and RP: 5’-CTTTGTGACCTGTTGAATCA-3’; interleukin (IL)-1β, FP: 5’-GGAGAAGAAGCAAGCAGAAAAT-CC-3’ and RP: 5’-TCCCATCTTCTCTTTGGATTG-3’; tumor necrosis factor (TNF)-α, FP: 5’-CTTCTGTCTACTGA-ACTTGGGT-3’ and RP: 5’TGGAAACGTGAGAGGAGGC-3’; iNOS, FP: 5’-GGAAGAGGAAACACTTGCTG- GT-3’ and RP: 5’-GAAACTGAGGGTACATGCTGAGC-3’; IL-6, FP: 5’-GTTATCCAGAGAGTGTAACGACAGC-3’ and RP: 5’-GCTTCAGATTTAGGAGGATGGG-3’; interleukin-10 (IL-10), FP: 5’-CTGCTATCGATTTCTCCCTGTGAG-3’ and RP: 5’TGA-GTGTCAAGCTAAGCTTATG-3’; intercellular adhesion molecule (ICAM-1), FP: 5’-GTCCTCTACTGTTGACG-3’; and RP: 5’TAAAGGGAATCTTCCGGCACCC-3’; vascular cell adhesion molecule (VCAM-1), FP: 5’-GA- CACCGTCATTATCTCTCGACT-3’ and RP: 5’-GTGTCGCGTAGGCTTCTATGC-3’. Immunoblotting SC tissues were homogenized in ice-cold lysis buffer (50 mm Tris-HCl, pH 7.4, containing 50 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 10% glycerol, and protease inhibitor mixture) and sample protein concentration was determined with Bradford reagent (Bio-Rad). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and immunoblotting were performed as described previously. Autoradiographs of immunoblots were generated by using the enhanced chemiluminescence detection kits (Amersham Biosciences, Arlington Heights, IL).

Enzyme-Linked Immunosorbent Assay (ELISA)

Anti-MBP-specific IgG isotypes were detected in serum samples by solid-phase ELISA. In brief, plates were coated with MBP (2 μg/ml) diluted in PBS overnight in a humidified chamber followed by washing with PBS containing 0.05% Tween 20 and blocking for 1 hour with 1% bovine serum albumin in PBS before the addition of serum samples. Samples were diluted 1:100 in PBS after 2 hours of incubation at room temperature and then plates were washed with PBS containing 0.05% Tween 20 to remove any unbound primary antibody. Bound antibody was detected by incubation with alkaline phosphatase-labeled rat anti-mouse IgG1, anti-mouse IgG2a and anti-mouse IgG2b (1:2000) from Sero-tec (Raleigh, NC) using p-nitrophenyl phosphate (Sigma-Aldrich) in 0.1 mol/L glycine buffer as a substrate. Absorbance was read at 405 nm in a microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT). Data are expressed as A405. NT-3 levels in serum samples were determined by using NT-3 Emax immunoassay kit (Promega, Madison, WI). Th2 cytokines IL-4 and IL-10 detected in SC tissue homogenates prepared in PBS by using a tissue homogenizer, Ultra-Turbax (IKA-Ultra Turrax, Staufen, Germany), and centrifuged at 12,000 g for 15 minutes at 4°C. IL-4 and IL-10 BD OptEIA ELISA kits (BD Biosciences, San Jose, CA) were used with the sandwich ELISA method (using the manufacturer’s protocol). Data were computed as concentration of cytokine/mg of SC tissue protein and plotted.

**Statistical Analysis**

Using Student's unpaired t-test and one-way analysis of variance (Student-Newman-Keuls: compare all pairs of columns), P values were determined for clinical score, real-time PCR analysis, ELISA, intensities, and counting data in triplicate from three independent experiments using GraphPad software (GraphPad Software Inc., San Diego, CA).

**Figure 1.** Lovastatin and AICAR in combination do not antagonize but complement each other in EAE animals. EAE was induced by immunization of female Lewis rats with MBP (50 μg s.c., emulsified in CFA), injected into the hind limb foot pad on days 0 and 7. Daily treatment with lovastatin (Lov, 1 mg/kg) and AICAR (Aic, 50 mg/kg) in combination or individually began from day 0 after immunization and continued until the end of study. Therapeutic dose of lovastatin (2 mg/kg) based on our previous observation was included for comparison analysis of combination treatment. Control rats received PBS (0.05 ml s.c., emulsified in CFA) injected into the hind limb foot and treated daily with placebo, CON (placebo) or combination of lovastatin and AICAR, CON (Lov + Aic). Clinical scores were observed at scale described under Materials and Methods. Three animals from each group were sacrificed 13 days after immunization (peak clinical day) and 25 days after immunization (recovered EAE rats). Data are representative of three identical experiments and presented as mean ± SD.
Results

Combination Therapy with Suboptimal Dose of Lovastatin and AICAR Complement Each Other in EAE

As we reported previously, to prevent the progression of EAE, optimal doses of lovastatin and AICAR were ≥2 mg/kg\textsuperscript{17,19,27} and ≥100 mg/kg\textsuperscript{22,29} respectively, when administered individually. Therefore, we first evaluated the therapeutic efficacy of the suboptimal dose of lovastatin (1 mg/kg) and AICAR (50 mg/kg) in combination or individually and compared those findings with their optimal dose effects. Combination treatment with suboptimal doses of lovastatin and AICAR initiated from 0 days after immunization with MBP prevented and delayed the onset of EAE in rats with greater efficacy than using the optimal dose of the individual drugs alone (Figure 1). Clinical signs of EAE were evident in placebo (vehicle)-treated and MBP-immunized rats from day 8 after immunization onwards, followed by acute disease resulting in 80 to 90% mortality (clinical score, ≥4.5) by 13 to 14 days after immunization (Figure 1). Combination treatment with lovastatin and AICAR prevented EAE disease severity (clinical score, 3.0) and normalized neurological functions in rats by 18 to 19 days after immunization (Figure 1). Conversely, the suboptimal dose of lovastatin lessened disease severity in EAE rats and enabled recovery, but this did not occur in animals treated with AICAR alone (mortality, 70 to 80%) (Figure 1). Corresponding with clinical symptoms, the body weight profile of EAE animals was improved by combination treatment with these drugs (data not shown). Importantly, there was no observed antagonism between AICAR and lovastatin: certain immunomodulatory agents reportedly antagonize one another’s effect.\textsuperscript{31}

Combination Therapy with Lovastatin and AICAR Reverses Pathological Changes and Cellular Infiltration in the CNS of EAE

Generally, MS treatment is initiated after patients have developed clinical signs or symptoms of CNS demyelination. Thus, it is essential to test whether a therapeutic regimen, which can prevent EAE induction, can effectively reverse an established case of EAE. Thus, we evaluated whether the combination of suboptimal doses could reverse established EAE. Treatment was initiated after the onset of disease when individual rats developed a clinical score of ≥2.0. No protection was detected in rats treated with lovastatin (1 mg/kg) or AICAR (50 mg/kg) alone after the onset of EAE (Figure 2A). As predicted, combination treatment with these drugs reversed EAE (Figure 2A).

As documented earlier,\textsuperscript{17} MS-associated pathological changes are usually evident in the lumbar region of the SC of EAE animals. Thus, we next evaluated the improvement in cellular infiltration and pathological changes in the lumbar SC of EAE rats. In association with clinical improvements, combination treatment reduced inflammation in the SC of EAE rats (Figure 2B). Inflammation observed in the SC of placebo-treated EAE rats was reduced in the different regions of transverse section of SC with combination treatment and not in those treated individually with the same dose (Table 1). The majority of CNS-infiltrating cells in MS/EAE comprises of CD4, CD8, and macrophages, so, we next quantified these cells in the transverse section of SC. There was an increase in number of ED1\textsuperscript{+} cells as a marker of activated macrophage and microglia (Figure 2, C and D) including both CD4 (Figure 2D) and CD8 T cells (Figure 2D) in the SC of placebo-treated EAE rats. Combination treatment with lovastatin and AICAR significantly reversed the infiltration of these infiltrating cells into the SC of EAE rats, compared with those treated individually with these drugs (Figure 2, C and D). Corresponding with these findings, CD4 and CD8 antigen protein level was decreased in the SC of EAE rats treated with the drug combination (Figure 2E). In addition, mRNA expression of antigen proteins for CD4, CD8, and CD11b (for macrophage and microglia) was significantly decreased in the SC of EAE rats treated with the drug combination, compared with placebo (Table 2). Because cellular infiltration involves interaction between the endothelium and mononuclear cells, mediated by expression of adhesion molecules (ICAM-1 and VCAM-1) and chemo-attractants (MCP-1 and its receptor CCR2), we next explored the effect of combination treatment on the restoration of endothelium functions. Similar to the cellular infiltration data, ICAM-1, VCAM-1, MCP-1, and CCR2 expression was decreased in the SC of EAE rats treated with the drug combination, compared with placebo (Table 2). Although the decrease in inflammatory mediator expression in the SC of EAE rats was significant when animals were treated with these drugs individually (compared with placebo), the drug combination was more impressive (Table 2). Together, these data document that the combination therapy with lovastatin and AICAR reverses pathological changes and cellular infiltration into the CNS of EAE animals.

Figure 2. Combination therapy with suboptimal dose of lovastatin and AICAR prevents or reverses clinical and histological changes and, attenuates cellular infiltration into the CNS of EAE animals. EAE was induced by immunization of female Lewis rats with MBP (50 μg s.c., emulsified in CFA), injected into the hind limb foot pad on days 0 and 7. A: Daily lovastatin (Lov, 1 mg/kg) and AICAR (Aic, 50 mg/kg) treatment in combination or individually began from 8 days after immunization (clinical score ≥2.0) and continued until the end of study. Animals were sacrificed 13 days after immunization to collect SC and fixed in 10% buffered formalin as described under Materials and Methods. SC sections were prepared and stained with H&E or by immunostaining. B: Representative sections of SC from each group depict infiltration of mononuclear cells into the CNS of EAE rats and its attenuation by treatment with drugs. Asterisks depict infiltration in the white matter region around the vessel. C: Representative sections demonstrate distribution of ED1\textsuperscript{+} cells (macrophage and microglia) in the SC of EAE animals. D: Pilot depicts manual counts of macrophages and microglia (ED1) and both helper (CD4) and cytotoxic (CD8) T cells infiltrated in the SC of EAE rats and their attenuation with the drug treatment. E: Representative autoradiograph exhibits level of CD4 and CD8 proteins in the SC of EAE rats treated with placebo or drugs. Data in plots are expressed as mean ± SD from three identical experiments. Statistical significances are indicated as *P < 0.05, **P < 0.01, ***P < 0.001, and NS (nonsignificant) versus EAE (placebo) by analysis of variance.
Combination Therapy with Suboptimal Doses of Lovastatin and AICAR Reverses Proinflammatory Response in the CNS of EAE Animals

The expression of proinflammatory mediators is reported to be elevated predominantly in the CNS of EAE animals during peak clinical day, which is associated with various pathological changes and neurological impairments. An abrupt increase in the expression of mRNA for proinflammatory cytokines, i.e., IFN-γ, TNF-α, and IL-1β, in the SC of placebo-treated EAE rats was significantly attenuated by combination treatment withLovastatin and AICAR (Table 2). Consistent with mRNA expression, the protein expression of IFN-γ was inhibited in the SC of EAE rats treated with the drug combination (Figure 3A). Furthermore, the expression of the NO-producing enzyme iNOS (mRNA and protein) was also attenuated in the SC of EAE rats with combination treatment, compared with placebo (Table 2 and Figure 3A). Notably, no significant decrease in the expression of these proinflammatory mediators was observed in the SC of EAE rats treated with AICAR alone, compared with placebo (Table 2). Treatment of EAE rats with lovastatin alone, however, attenuated the expression of these proinflammatory mediators compared with placebo, but this was not as profound as that observed in EAE rats treated with the drug combination (Table 2). Together, these data reveal that combination therapy of lovastatin and AICAR attenuates proinflammatory immune response that is vital for the establishment of EAE.

Combination Therapy with Lovastatin and AICAR Promotes Expression of Anti-Inflammatory Immune Response in the CNS of EAE Animals

Because the anti-inflammatory immune response plays an important role for the attenuation of EAE, we next determined the expression of these mediators in the SC.

Table 1. Clinical Scores and Cellular Infiltration in the Spinal Cord of EAE Animals Treated with Lovastatin and AICAR in Combination or Individually at the Same Doses

<table>
<thead>
<tr>
<th>Groups</th>
<th>Clinical score (mean ± SD)*</th>
<th>Subarach infiltrates (n = 9)** ‡</th>
<th>Perivascular infiltrates (n = 9)** ‡</th>
<th>Cuffing (n = 9)** ‡</th>
<th>Parenchymal infiltrates (n = 9)** ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON (placebo)†</td>
<td>0 ± 0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>CON (Lov + Aic)†</td>
<td>0 ± 0</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>EAE (placebo)†</td>
<td>3.83 ± 0.28</td>
<td>+++</td>
<td>+++</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>EAE (Aic)†</td>
<td>3.73 ± 0.28</td>
<td>+++</td>
<td>++</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>EAE (Lov)†</td>
<td>3.4 ± 0.29</td>
<td>++</td>
<td>+</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>EAE (Lov + Aic)†</td>
<td>2.5 ± 0.5</td>
<td>+</td>
<td>+</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

*EAE was induced by immunization of female Lewis rats with MBP (50 µg s.c., emulsified in CFA), injected at the hind limb foot pad on days 0 and 7. †Placebo orlovastatin (1 mg/kg) and AICAR (50 mg/kg) was administered intraperitoneally in combination or individually after the onset of EAE (8 dpi onwards; clinical score ≥2.0). **Infiltration in the different regions of SC section was scored as; +, slight; ++, moderate; ++++, intense. Y, positive; N, negative. Numbers in parentheses indicate SC sections examined from animals with the similar clinical score.

Table 2. The mRNA Expression Profile of Proinflammatory and Anti-Inflammatory Mediators in the Spinal Cord of EAE Animals Treated with Lovastatin or AICAR in Combination or Individually

<table>
<thead>
<tr>
<th>Groups</th>
<th>CON (placebo)</th>
<th>CON (Lov + Aic)</th>
<th>EAE (placebo)</th>
<th>EAE (Aic)</th>
<th>EAE (Lov)</th>
<th>EAE (Lov + Aic)</th>
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<tbody>
<tr>
<td>Mononuclear cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD4</td>
<td>0.22 ± 0.1</td>
<td>0.25 ± 0.12</td>
<td>9.8 ± 0.06</td>
<td>7.5 ± 0.2NS</td>
<td>6.16 ± 0.45*</td>
<td>4.6 ± 0.3§</td>
</tr>
<tr>
<td>CD8</td>
<td>0.2 ± 0.02</td>
<td>0.15 ± 0.03</td>
<td>4.8 ± 0.12</td>
<td>3.9 ± 0.05NS</td>
<td>2.3 ± 0.4*</td>
<td>1.2 ± 0.2§</td>
</tr>
<tr>
<td>CD11b</td>
<td>0.45 ± 0.16</td>
<td>0.29 ± 0.13</td>
<td>33 ± 4.5</td>
<td>28 ± 2.9NS</td>
<td>15.3 ± 1.9†</td>
<td>5.7 ± 1.4§</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>ICAM-1</td>
<td>4.4 ± 0.35</td>
<td>3.12 ± 0.43</td>
<td>174 ± 12.25</td>
<td>105 ± 2.4NS</td>
<td>55.2 ± 2.6*</td>
<td>35 ± 1.5§</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>6.5 ± 0.43</td>
<td>4.32 ± 0.32</td>
<td>91.0 ± 10.2</td>
<td>50.2 ± 4.3*</td>
<td>31.1 ± 2.22†</td>
<td>2.5 ± 1.5§</td>
</tr>
<tr>
<td>Proinflammatory cytokines (Th1 cytokines)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IFN-γ</td>
<td>1.26 ± 0.4</td>
<td>0.97 ± 0.21</td>
<td>9.34 ± 2.35</td>
<td>7.6 ± 2.42NS</td>
<td>6.57 ± 1.42*</td>
<td>3.4 ± 1.6§</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.43 ± 0.2</td>
<td>0.63 ± 0.05</td>
<td>18.7 ± 1.52</td>
<td>16.7 ± 3.3NS</td>
<td>12.7 ± 2.08*</td>
<td>7.3 ± 1.5§</td>
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<tr>
<td>TNF-α</td>
<td>0.4 ± 0.06</td>
<td>0.9 ± 0.2</td>
<td>82.4 ± 10.2</td>
<td>72.3 ± 18NS</td>
<td>59.3 ± 12.1*</td>
<td>10 ± 1.4§</td>
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<tr>
<td>iNOS</td>
<td>0.65 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>120 ± 26.9</td>
<td>96.5 ± 3.4NS</td>
<td>60.2 ± 3.16§</td>
<td>14 ± 0.3§</td>
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<tr>
<td>Anti-inflammatory cytokines (Th2 cytokines)</td>
<td></td>
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<tr>
<td>IL-4</td>
<td>0.22 ± 0.2</td>
<td>1.44 ± 0.86</td>
<td>0.89 ± 0.08</td>
<td>0.61 ± 0.47NS</td>
<td>2.11 ± 0.51</td>
<td>3.8 ± 1.6§</td>
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<tr>
<td>IL-10</td>
<td>0.52 ± 0.2</td>
<td>1.28 ± 0.62</td>
<td>0.22 ± 0.01</td>
<td>0.42 ± 0.05NS</td>
<td>3.6 ± 0.33†</td>
<td>7.9 ± 0.4§</td>
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<tr>
<td>Chemokines</td>
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<tr>
<td>MCP-1</td>
<td>2.93 ± 0.2</td>
<td>3.38 ± 0.2</td>
<td>14.3 ± 1.7</td>
<td>8.98 ± 0.3*</td>
<td>6.4 ± 1.63*</td>
<td>4.4 ± 0.9§</td>
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<tr>
<td>CCR2</td>
<td>1.11 ± 0.3</td>
<td>1.18 ± 0.15</td>
<td>39.5 ± 9.08</td>
<td>30.9 ± 2.17*</td>
<td>7.04 ± 1.71†</td>
<td>1.7 ± 0.3§</td>
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</table>

The mRNA expression was determined by real-time QPCR and normalized with control gene (GAPDH) expression. Results are presented as mean ± SD from three identical experiments. Statistical significances are indicated as *P < 0.05, †P < 0.01, and ‡P < 0.001 versus EAE (placebo), and NS (nonsignificant) versus EAE (placebo) by one-way ANOVA.
Combination treatment withLovastatin and AICARinduced a significant increase in the expression of bothIL-4 and IL-10 (mRNA and protein) in the SC of EAE rats when compared with those treated with placebo on both days 13 and 25 after immunization (Table 2 and Figure 3, C and D). The expression of these cytokines (mRNA and protein) was however elevated significantly in the SC of EAE rats treated withLovastatin alone, compared with placebo, but this increase was not that profound as that observed in EAE rats treated with the drug combination (Table 2 and Figure 3, C and D). No significant increase in expression of these mediators was observed in the SC of EAE rats treated withAICAR alone, compared with placebo (Table 2 and Figure 3, C and D). Together, these data showed that combination therapy withLovastatin andAICAR promotes strong anti-inflammatory immune response in the CNS to reverse the established EAE.
Combination Therapy with Lovastatin and AICAR Promotes Induction of Myelin-Reactive Th2 Cells

Our data imply that the combination treatment with sub-optimal dose of lovastatin and AICAR may additively modulate some aspects of T-cell function in vivo. The reduction in CNS inflammation observed in EAE rats when treated with the drug combination is suggestive of its potential for selective reduction in the Th1 cell reactivity that primarily mediates the immune response generally associated with EAE pathogenesis. If this is the case, a consequence of lovastatin and AICAR treatment may be a bias toward Th2 activity, which is believed to protect against the development of EAE. Because the Th1 responses predominantly elicit IgG2a, whereas Th2 responses produce higher levels of IgG1 in mice, we assessed whether combination treatment influences the pattern of isotypes of MBP-specific antibodies after immunization with MBP. The elevated level of IgG2a isotype immunoglobulin detected in the sera of EAE rats was significantly reduced in EAE rats treated with the drug combination on days 13 and 25 after immunization (Figure 4A). Conversely, immunoglobulin isotype IgG1 (Figure 4B) and IgG2b (Figure 4C) were significantly increased in the sera of EAE rats treated with the drug combination, compared with placebo. No significant difference was observed in IgG2a levels in the sera of EAE rats treated with lovastatin or AICAR alone when compared with those treated with placebo (Figure 4A). IgG1 and IgG2b levels were significantly elevated in EAE rats treated with lovastatin but not in those treated with AICAR alone, compared with placebo-treated EAE rats (Figure 4, B and C). These data indicate that combination therapy with lovastatin and AICAR biases the anti-myelin protein immunoglobulin response toward IgG1 and IgG2b and against IgG2a, indicative of a Th1-to-Th2 shift.

Combination Therapy with Lovastatin and AICAR Alleviates Neurodegeneration in the CNS of EAE Animals

Because CNS inflammation-induced demyelination is associated with loss of both neuronal axons and oligodendrocytes resulting in the development of neurological deficits in MS/EAE, we next evaluated neuroprotection by histological examination of SC transverse sections for demyelination and axonal loss. In association with inflammation, LFB staining revealed increased demyelination with corresponding increase in cellular infiltration in the SC of EAE rats (Figure 5, A and B). Conversely, combination treatment reversed both cellular infiltration and demyelination in the SC of EAE rats (Figure 5, A and B). However, cellular infiltration and demyelination were significantly attenuated in the EAE rats treated with lovastatin or AICAR individually, compared with placebo, but these effects were not as profound as those resulting from combination treatment (Figure 5, A and B). Furthermore, immunohistochemistry for myelin proteins ie, MBP and MAG further corroborated these data and showed a significant reduction of demyelination in the SC of EAE rats treated with the drug combination (Figure 5, C and D). This reduction of demyelination was significantly less in the SC of EAE rats treated with lovastatin or AICAR alone when compared with placebo, but it was not as significant as observed with combination treatment (Figure 5, C and D).

Silver impregnation of axons and immunostaining for neuronal axon-specific protein, phosphorylated-neurofilament heavy chain (SMI-31), revealed a greater loss of axons in the white matter (Figure 6, A and B) as well as gray matter (data not shown) of the SC of EAE rats, which was attenuated by combination treatment with lovastatin and AICAR. The extent of this loss of axons was also greater in EAE rats treated with lovastatin or AICAR alone.

Figure 4. Combination therapy with lovastatin and AICAR promotes secretion of MBP-specific immunoglobulin biased toward Th2-type response in the sera. Myelin (MBP)-specific immunoglobulin levels were detected in the sera of EAE rats treated with combination of lovastatin and AICAR after the onset of disease after immunization with MBP by ELISA as described under Materials and Methods. Plots demonstrate alterations in the level of MBP-specific immunoglobulin IgG2a (A), IgG1 (B), and IgG2b (C) in the sera. Data in plots are expressed as mean ± SD from three identical experiments. Statistical significance was shown as *P < 0.05, **P < 0.01, ***P < 0.001, and NS (nonsignificant) versus EAE (placebo) by one-way analysis of variance.
Figure 5. Combination therapy with lovastatin and AICAR lessens demyelination in the CNS of EAE animals. SC sections of rats sacrificed at 13 days after immunization were fixed in 10% buffered formalin and stained with Luxol fast blue and hematoxylin (LFB) or by immunostaining as described under Materials and Methods. A: Nuclei counts revealed association of cellular infiltration with myelin break-down in the white matter region of SC of EAE rats and its reversal with the drug treatment. B: Representative sections show demyelination with corresponding increase in cellular infiltration (asterisk) in the white matter lesion of SC. Further, double immunostaining for MBP and MAG was performed to confirm LFB staining. C: Plot exhibits intensities for MBP and MAG in the white matter region of SC of EAE rats treated with the drug combination or alone. D: Representative sections show decrease (arrowheads) in immunostaining for MBP and MAG in the white matter region of SC of EAE rats and protection with the drug combination. Intensity data are presented as random units (R.U.). Data in plots are expressed as mean ± SD of SC sections from three identical experiments. Statistical significances are indicated as *P < 0.05, **P < 0.01, ***P < 0.001 versus EAE (placebo) by one-way analysis of variance.
(Figure 6, A and B). Corresponding with histological examinations, quantification of axons and immunofluorescence intensity for SMI-31 also corroborated these observations (Figure 6C). Because NT-3 (neurotrophic factor) is an important factor for the survival of neurons, we next measured the effect of combination treatment on
NT-3 secretion in EAE rats. Corresponding with axonal loss data, the observed decrease in level of NT-3 in the sera of EAE rats was significantly impeded by combination treatment (Figure 6D). Conversely, no significant change in NT-3 was observed in the EAE rats treated with lovastatin or AICAR alone when compared with placebo (Figure 6D). Together, these data reveal that combination therapy with lovastatin and AICAR preserves axons and myelin sheath against inflammation-associated neurodegeneration in established EAE.

Discussion

Combination therapies with existing or novel MS therapeutics are increasingly recognized as having great clinical potential for improving MS treatment outcomes.\(^{15}\) Combination therapies have been tested in animals\(^ {30,34}\) and humans,\(^ {35}\) including one phase I clinical trial\(^ {36}\) with major Food and Drug Administration-approved immunomodulators in the MS regimen. Moreover, other combination therapies are also being investigated in MS with Food and Drug Administration-approved therapies, ie, subcutaneous IFN-\(\gamma\)1a, intramuscular IFN-\(\beta\)-1a, subcutaneous IFN-\(\beta\)-1b, glatiramer acetate, and mitoxantrone.\(^ {37,38}\) Combination therapy for MS is advantageous if both drugs 1) have different mechanisms of actions, 2) have excellent safety profiles, and 3) have no additional toxicities when used in combination for additive or synergistic effects. In this regard, statins and AICAR meet these criteria and both characteristically down-regulate the expression of multiple mediators associated with induction of EAE.\(^ {16,17,19,22}\) Recent open small scale clinical trials of simvastatin and atorvastatin hold promise to prevent the progression of autoimmune diseases such as MS\(^ {18}\) and rheumatoid arthritis.\(^ {20}\) Respectively. Previous studies indicate that statins mediate immunomodulatory effects via inhibiting synthesis of isoprenoid compounds in the mevalonate pathway, which is important for isoprenylation of small G-proteins (Ras/Rho A) essential for proinflammatory signaling events\(^ {39}\) such as intracellular trafficking and subcellular localization to the cytoplasmic surface of the plasma membrane.\(^ {40}\) Similarly, AICAR-induced immunomodulatory effects are mediated via activation of AMPK as evidenced from our recent studies, ie, the attenuation of lipopolysaccharide-induced nuclear factor (NF)-\(\kappa\)B activation and NF-\(\kappa\)B kinase activity in CNS glial cells.\(^ {23}\) and the attenuation of relapsing-remitting EAE.\(^ {22}\) We believe that lovastatin and AICAR are excellent therapeutic candidates for MS or other related CNS demyelinating diseases.

This study establishes that combination therapy with lovastatin and AICAR have synergistic or additive effects for the prevention or reversal of EAE, more so than optimal doses of either drug alone. More importantly, combination therapy with suboptimal doses of these drugs reversed paralysis when daily treatment was started after EAE was established, and this was not achieved with either agent alone. In addition, combination therapy provided neuroprotection in remitting EAE. Furthermore, combination therapy with these drugs reduced cellular infiltration and pathological changes in established EAE. In line with these findings, combination therapy down-regulated proinflammatory immune responses with parallel induction of anti-inflammatory immune responses and biased MBP-specific IgG2a toward IgG1 and IgG2b, indicative of a Th1-to-Th2 shift. Lovastatin and AICAR used individually at suboptimal doses did not markedly decrease disease severity. However, the drug combination prevented and reversed established EAE when evaluated clinically or by neuropathological criteria.

Although other mechanisms may contribute to the additive or synergistic effect of lovastatin and AICAR in combination, we suggest that the immunomodulatory effect of these drugs is complementary. Previous studies document that lovastatin\(^ {17,19}\) and atorvastatin\(^ {16}\) attenuate both acute and remitting-relapsing EAE in experimental MS models by promoting Th2-biased immune responses. In gene array-based studies, lovastatin selectively down-regulated various immune response genes associated with the progression of acute EAE.\(^ {17}\) Earlier studies suggest that statins 1) attenuate inducible MHC class II expression on nonprofessional antigen-presenting cells through IFN-\(\gamma\);\(^ {41}\) 2) down-regulate Rho-mediated migration of monocytes across the brain endothelium by altering isoprenoid biosynthesis;\(^ {42}\) and 3) inhibit expression of adhesion molecules and matrix metalloproteinase such as MMP-9, a key event in crossing the blood-brain-barrier and postdiapedesis parenchymal transmigration.\(^ {43,44}\) Our laboratory showed that modulation of the Th1/Th2 axis in a remitting-relapsing EAE model of MS by AICAR may be mediated via AMPK activation.\(^ {22}\) AICAR-induced activation of AMPK is reported to inhibit the NF-\(\kappa\)B-dependent expression of adhesion molecules (ICAM-1 and VCAM-1) in human umbilical vein endothelial cells.\(^ {45}\) Likewise, anti-inflammatory properties of AICAR protects against injury during cardiac ischemia and reperfusion in transplanted rat heart\(^ {46}\) and dog kidney\(^ {47}\) as well as in chest trauma and inflamed tissues.\(^ {48,49}\)

Recently, we documented the restoration of the remyelination process in the CNS of lovastatin-treated EAE animals via enhanced survival and differentiation of oligodendrocyte progenitors,\(^ {21}\) but its effect on the survival and differentiation of neuronal axons in vivo deserve detailed investigation. However, the protection of cortical neurons by different statins against excitotoxicity induced cell death has already been shown \textit{in vitro}.\(^ {50}\) Also, AMPK activation-mediated enhanced survival of hippocampal neurons has been documented \textit{in vitro} under conditions of reduced energy availability during glucose deprivation and glutamate excitotoxicity.\(^ {26}\) In addition, the activation of AMPK has been shown to inhibit both apoptotic and necrotic death of both astrocytes\(^ {51}\) and thymocytes.\(^ {52}\) Therefore, we deduce from our data that the attenuation of inflammation-associated neurodegeneration in part is attributed to both lovastatin and AICAR. Together, our data suggest that combination therapy with lovastatin and AICAR alleviate inflammation-associated neurodegeneration in the CNS of EAE animals.

At this time, the precise contributions by which combination therapy with suboptimal doses of lovastatin and AICAR reverse established EAE are not fully understood. The increased efficacy of the suboptimal dose of lovastatin and AICAR in combination may be attributed to
their synergistic or additive effects within the CNS to alleviate neurodegeneration. The attenuation of neuroinflammation and the decrease in both axonal loss and demyelination would be consistent with this postulate. Although the animal model used in the study develops acute EAE that mimics acute MS, but similar results were also observed when tested in relapsing-remitting model of MS (A.S. Paintlia, M.K. Paintlia, I. Singh, and A.K. Singh, manuscript in preparation). Our findings suggest a future clinical trial is warranted to investigate combination therapy of lovastatin and AICAR in MS or other CNS neuroinflammatory diseases.

Acknowledgments

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