5-Aminoimidazole-4-Carboxamide Ribonucleoside: A Novel Immunomodulator with Therapeutic Efficacy in Experimental Autoimmune Encephalomyelitis

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5-Aminoimidazole-4-Carboxamide Ribonucleoside: A Novel Immunomodulator with Therapeutic Efficacy in Experimental Autoimmune Encephalomyelitis

Narender Nath,* Shailendra Giri,* Ratna Prasad,* Mohamad Labib Salem,‡ Avtar K. Singh,‡ and Inderjit Singh2*

Experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, is a Th1-mediated inflammatory demyelinating disease of the CNS. AMP-activated protein kinase was reported recently to have anti-inflammatory activities by negatively regulating NF-κB signaling. In this study, we investigated the prophylactic and therapeutic efficacy of an AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), in active and passive EAE induced by active immunization with PLP139–151 or MOG35–55 and in adoptive transfer of PLP139–151-sensitized T cells, respectively. In vivo treatment with AICAR exerted both prophylactic and therapeutic effects on EAE, attenuating the severity of clinical disease. The anti-inflammatory effects of AICAR were associated with the inhibition of the Ag-specific recall responses and inhibition of the Th1-type cytokines IFN-γ and TNF-α, whereas it induced the production of Th2 cytokines IL-4 and IL-10. Treatment of PLP139–151-specific T cells in vitro with AICAR decreased their expression of T-bet in response to IL-12, a Th1 transcription factor, whereas in response to IL-4, it induced the expression and phosphorylation of Th2 transcription factors GATA3 and STAT6, respectively. Moreover, treatment of APCs in vitro with AICAR inhibited their capability to present the proteolipid protein peptide to PLP139–151-specific T cells. In an irrelevant Th1-mediated, OT-2 TCR transgenic mouse model, AICAR impaired in vivo Ag-specific expansion of CD4+ T cells. Together, these findings show for the first time that AICAR is a novel immunomodulator with promising beneficial effects for the treatment of multiple sclerosis and other Th1-mediated inflammatory diseases.

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Multiple sclerosis (MS) is an inflammatory disease of the CNS, characterized by perivascular cuffs of mononuclear cells that include both lymphocytes and macrophages (MΦ), which lead to the damage of myelin sheaths and the underlying axons (1, 2). The etiology of MS remains unknown, but the composition of plaques, immunogenetic background response to immunomodulation, and data from animal models suggest that it is an autoimmune disease mediated by myelin-specific CD4+ and CD8+ T cells (3, 4). Already, chemotherapy using immunosuppressive drugs such as mitoxanthrone and cyclophosphamide or immunotherapy using immunomodulatory drugs such as IFN-β, glatiramer acetate, and statins are being used or are in clinical studies for the treatment of MS (5–7). AMP-activated protein kinase (AMPK) is a multisubstrate protein kinase that plays a central role in the response to metabolic stress in protecting the cell against ATP depletion by inhibiting biosynthetic pathways and stimulating energy-generating pathways (8–10). AMPK phosphorylates enzymes of lipid metabolism such as HMG-CoA reductase, acetyl CoA carboxylase, and hormone-sensitive lipase and thereby regulates lipid metabolism (11). 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) has been studied extensively as an activator of AMPK in different cell types (12–14). Once inside the cell, AICAR is phosphorylated by adenosine kinase to ZMP, which mimics AMP and activates AMPK without altering the cellular levels of ATP, ADP, or AMP (13). Activation of AMPK has been reported to be associated with a variety of beneficial effects on the cardiovascular system, including increasing the regional blood flow and local adenosine concentration (15), inhibiting neutrophil activation (16), suppressing platelet activation and intracoronary thrombosis (17), preventing oxidant-induced injury (18), and potentiating the protective mechanisms induced by myocardial preconditioning (19). Several clinical trials have been conducted to evaluate the beneficial effect of AICAR in preventing adverse effects of coronary artery bypass grafting surgery, and the results have been reported to be significant (20–22). Recently, we have demonstrated that AICAR inhibits the production of proinflammatory mediators (TNF-α, IL-1β, IL-6, and NO) in primary astrocytes, microglia, and MΦ via inhibition of NF-κB and the CREB pathways (23). We have also reported that AICAR attenuates LPS-induced inflammation in rats (23). With these pharmacological properties, we hypothesized that AICAR might exert anti-inflammatory effects on autoimmune diseases. To address this hypothesis, we designed the present study to define the impact of AICAR treatment on experimental autoimmune encephalomyelitis (EAE) and the nature of associated immune responses. We found that AICAR exerted both prophylactic and therapeutic effects on...
the clinical disease in active and passive EAE. The anti-inflammatory effects of AICAR were mediated, at least in part, by immune-mediated responses. AICAR inhibited the Th1-type response and Ag presentation, whereas it induced the Th2-type response, suggesting that both T cells and APCs are targets for AICAR. To our knowledge, this is the first report to show the inhibitory effect of AICAR on EAE, opening a new avenue for the treatment of MS and other Th1-mediated inflammatory diseases.

Materials and Methods

Mice

Female 4- to 5-wk-old SJL and C57BL/6 mice were obtained from The Jackson Laboratory. OT-2 TCR transgenic mice on a C57BL/6 background were obtained from The Jackson Laboratory. OT-2 mice (Ly5.2+) were transferred i.v. into congenic C57BL/6 (Ly5.1−) mice. Two days after transfer, the mice were s.c. immunized with 100 μg of OVA323–339 peptide in CFA. The final concentrations of peptide and antigen were 100 and 200 μg per mouse, respectively. Each group consisted of 5 mice.

Peptide and reagents

Myelin proteolipid protein peptide (PLP139–151) (HSLGTKWLGHPDKF) and MOG35–55 (MEGVYWPSRFVSVVLRNGK) were purchased from Peprotech International, and OVA223–239 (ISQAVHAAHHEINAEGR) was purchased from American Peptide. AICAR was purchased from Toronto Chemicals. Recombinant murine IL-2, IL-4, and IL-12 were purchased from BD Pharimingen. Anti-mouse CD4 (PE; GK1.5), CD45.2 (FITC; 104), and CD16/CD32 (F(ab′)2 anti-mouse) were from BD Biosciences. The anti-CD4 and anti-CD8 Abs were purchased from Zymed Laboratories, and GATA3 Abs were purchased from Santa Cruz Biotechnology.

Active EAE induction in SJL/J and C57BL/6 mice

Mice were immunized on day 0 by s.c. injections in the flank region with 100 μl of an emulsion of PLP139–151 (SJL/J) and MOG35–55 (C57BL/6) peptide in CFA. The final concentrations of peptide and Mycobacterium tuberculosis H37Ra were 100 and 200 μg per mouse, respectively. Each group consisted of 5 mice.

Adoptive transfer of PLP139–151 peptide-specific T cells for passive EAE induction

Female donor mice (6- to 10-wk-old) were s.c. immunized with an emulsion (200 μl) containing 100 μg of myelin PLP139–151 and 200 μg of M. tuberculosis H37Ra peptide in CFA. The final concentrations of peptide and M. tuberculosis H37Ra were 100 and 200 μg per mouse, respectively. Each group consisted of 5 mice.

Histology

To assess the infiltration of immune cells in the CNS, the SJL/J mice in the treated and untreated groups were killed on day 13 (at the peak of the disease) and on day 60 (relapsing stage of EAE) by CO2 asphyxiation. The lumbar regions of the spinal cord were taken out and stored in 10% buffered formalin. Paraffin-embedded 4-μm-thick transverse sections of the mouse spinal cord (six sections per mouse) were stained with H&E to assess leukocyte infiltration and inflammation using an Olympus digital camera (Olympus BX-60). On day 13, the spinal cord sections from five representative animals (untreated and AICAR treated) were processed and stained with Hoescht 33342 dye (for nuclei stain). An image of the artery, periphery, and center regions were taken by fluorescent microscopy. Five random fields were taken for periphery and center. Hoechst-positive cells were counted by Image Pro-Plus 4.0 software (Media Cybernetics) and plotted as fold change in infiltration during the disease and treatment process (24).

Recall responses

Myelin PLP139–151-immune spleen cells (2 × 105/100 μl/well) were cultured in 96-well, round-bottom microculture plates (Falcon Labware) in 0.1 ml of RPMI-complete in the presence of 1–10 μg/ml PLP139–151 peptide and AICAR (0.1–1.0 mM). For evaluation of the T cell recall responses in the SJL/J EAE model, spleens were harvested at day 60. Spleen cell suspensions in which RBCs had been lysed were plated at a density of 5 × 106 cells/well in a 96-well plate in RPMI 1640 in the presence or the absence (background) of varying concentrations of PLP139–151. For proliferation, [3H]thymidine (1 μCi/well) was added at 48 h, and mean incorporation of thymidine in DNA was measured after 72 h by 1450 MicroWallac Trilux Liquid Scintillation Counter (Perkin-Elmer Life Sciences).

Effect of AICAR on Th1 and Th2 cytokines in PLP139–151 sensitized T cells

Myelin PLP139–151-immune spleen cells (5 × 106/ml) were cultured in RPMI-complete in 24-well plates with 5 μg/ml PLP139–151 in the presence of AICAR (0.1–1.0 mM). The culture supernatants were collected at different time points (48 and 120 h) and checked for cytokine production. The level of Th1 (IFN-γ and TNF-α) and Th2 (IL-4) cytokines in culture supernatants was measured by the ELISA OPTEIA system (BD Pharimingen) as per the manufacturer’s instructions.

Western blot analysis

Cells were incubated in the presence or absence of different stimuli, harvested, washed with Hanks’ buffer, and sonicated in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (1 mM PMSF, 5 μl/ml aprotinin, 5 μg/ml anti-trypsin, 5 μg/ml leupeptin). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was then blocked for 1 h in 5% nonfat dry milk, TBS (20 mM Tris, 500 mM NaCl, and 1% Tween-20), pH 7.4, and unoccupied overnight in primary antisera (pSTAT4/STAT4, pSTAT6/STAT6, and T-bet) containing 5% nonfat dry milk or 5% BSA in case of phosphoantibodies. Blots were washed with TBS (four times, 5 min) and incubated for 45 min at room temperature. HRP-conjugated anti-rabbit or anti-mouse secondary Ab was added at a dilution of 1:5000. The blots were washed three times in TBS and developed with an ECL detection system (23, 25).

Coculture of AICAR-treated Mφ with PLP139–151-specific T cells

The peritoneal Mφ were isolated from naive SJL/J mice as described previously (23). The cells were pretreated with AICAR (0.1–1.0 mM) and cocultured with PLP139–151-specific T cells in the presence of PLP139–151 (5 μg/ml) for 96 h. Cocultures were pulsed with 1 μCi/well [3H]ThiDr for the final 24 h of the 96-h incubation period. The [3H]ThiDr uptake was detected as above. The long-term PLP139–151-specific T cell line was derived from SJL/J mice primed with PLP139–151, as described previously (25).

In vivo Ag-specific OT-2 CD4 T cell responses

For evaluation of Ag-specific CD4 T cell expansion in vivo, OT-2 TCR transgenic mice were used as a source of OT-2 peptide-specific (OVA223–239-specific) donor CD4+ T cells. The spleen cells were harvested from OT-2 mice (Ly5.2+), prepared as a single-cell suspension, and lysed with 1% Pharma Lyse (BD Pharimingen) to remove RBCs. Subsequently, 3 × 5 × 106 spleen cells in 0.3 ml of PBS were adaptively transferred i.v. into congenic C57BL/6 (Ly5.1−) mice. Two days after transfer, the mice were s.c. immunized with 100 μg of OT-2 peptide OVA223–239 in PBS. Immunized mice were subsequently treated i.p. with PBS or AICAR (100 or 500 mg/kg body weight). The frequency of OT-2
CD4⁺ T cells was determined in recipient mice by staining with FITC-labeled Ly5.2 and PE-labeled CD4 (BD Pharmingen) on days 3, 5, and 7 in PBLs and on day 7 in DLNs (26).

Statistical analysis

Clinical disease scores are presented as the average maximal scores over the treatment period (mean ± SD) and were analyzed using the Kruskal-Wallis test, a nonparametric alternative to the one-way ANOVA. Log-rank tests were used to assess the difference in time to disease induction (disease score, >0; Kaplan-Meier Survival Curve). Statistical significance was set at 0.05. All analyses were conducted using SAS statistical software (version 8). Statistics for proliferation and cytokine responses were analyzed with one-way multiple-range ANOVA GraphPad Prism 3.0 software. Significances (p value) between groups were determined using the Newman-Keuls test. A value of p < 0.05* and above was considered significant.

Results

In vivo treatment with AICAR-induced prophylactic and therapeutic effects on EAE

To induce EAE, the SJL/J mice were immunized with myelin PLP₁₃₉₋₁₅₁ peptide (active disease) or by adoptive transfer of myelin-PLP₁₃₉₋₁₅₁-sensitized T cells. Passive EAE was induced in naive SJL/J mice by adoptive transfer for 60 days to define its prophylactic effects (a–c). For therapeutic efficacy, the AICAR was injected from day 14 (d) in active and day 10 (e) in adoptive EAE in SJL/J mice. The disease is represented here as the mean score throughout the cohort, and results are summarized in Table I.

Table I. Efficacy of AICAR in EAE*

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Peptide of Immunization</th>
<th>Treatment with AICAR</th>
<th>Incidence of EAE</th>
<th>Mean Disease Score and Area Under Curve</th>
<th>Day of Disease Onset</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Active Area Passive</td>
<td>Active Passive</td>
</tr>
<tr>
<td>SJL/J</td>
<td>PLP₁₃₉₋₁₅₁</td>
<td>None</td>
<td>22/22</td>
<td>3.1 ± 0.1 29.77 3.3 ± 0.3 155.4</td>
<td>13.0 ± 0.8 8.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mg/kg</td>
<td>18/21</td>
<td>1.85 ± 0.6 19.58 2.5 ± 0.6 74.15</td>
<td>14.7 ± 1.5 9.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 mg/kg</td>
<td>17/18</td>
<td>1.52 ± 0.5 6.33 1.7 ± 0.4 24.25</td>
<td>14.3 ± 0.6 11.0 ± 0.0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>MOG₅₅₋₅₅</td>
<td>None</td>
<td>6/6</td>
<td>2.69 ± 0.07 46.98</td>
<td>11.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mg/kg</td>
<td>6/6</td>
<td>1.83 ± 0.14 27.61</td>
<td>12.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500 mg/kg</td>
<td>6/6</td>
<td>1.25 ± 0.41 21.33</td>
<td>14.0 ± 1.4</td>
</tr>
</tbody>
</table>

* Clinical disease scores are presented as the average maximal scores over the treatment period (mean ± SD) and analyzed using the Kruskal-Wallis test.
mice, starting on days 14 and 10 (the time point at which the clinical disease is established), respectively. AICAR treatment at 100 or 500 mg/kg decreased the clinical severity of EAE disease at both doses in active EAE (EAE, 2.93 ± 0.19; AICAR: 100 mg/kg, 2.7 ± 0.3; 500 mg/kg, 2.7 ± 0.27; area under the curve of 27.94, 22.7, and 20.20; p < 0.05) and passive EAE (EAE, 2.13 ± 0.85; AICAR: 100 mg/kg, 1.25 ± 1.10; 500 mg/kg, 1.5 ± 1.0; area under the curve of 49.98, 23.88, and 34.69; p < 0.05) from the first peak of disease (Fig. 1, d and e). Although AICAR doses <100 mg/kg (25 or 50 mg/kg) induced some protection, they did not show significant effects on EAE (data not shown).

AICAR limits the infiltration of immune cells to the CNS

The hallmark of EAE is the infiltration of inflammatory cells into the CNS, leading to tissue damage. Therefore, we aimed to investigate the effect of AICAR on the infiltration of the inflammatory cells into the CNS of untreated and treated EAE animals. As shown in Fig. 2b, the untreated EAE mice showed profound inflammation in the CNS. However, treatment with either 100 mg/kg (Fig. 2c) or 500 mg/kg (d) AICAR doses significantly reduced the infiltration.

FIGURE 2. The protective effects of AICAR therapy on EAE associates with inhibition of infiltration of inflammatory cells into the CNS. Adoptive EAE was induced in SJL/J mice, and AICAR treatment was done as described in the legend to Fig. 1. Spinal cords (lumbar regions) were harvested from the naive mice (a) and EAE mice (b), treated with 100 mg/kg AICAR (c) or 500 mg/kg AICAR (d) on day 13 after the adoptive transfer of PLP139–151-specific T cells for EAE induction. Tissues were fixed (10% buffered formalin) and embedded in paraffin, and the paraffin sections were stained with H&E and visualized at ×10 to determine infiltration. e–g, Hoechst-positive cells were counted on day 13 in spinal cord sections of naive (1), EAE (2), EAE + A100 (3), and EAE + A500 (4) mice and plotted for the artery, periphery, and center regions. ###, p < 0.001 compared to naive; *, p < 0.05, **, p < 0.01, and ***, p < 0.001 compared to EAE.

Errors of MMCSs in all treated groups with active and passive EAE and area under the curves are summarized in Table I.

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FIGURE 3. The protective effect of AICAR on EAE coincides with induced production of IL-10. The spleen cells were harvested from naive (unimmunized) SJL/J mice, or from untreated EAE mice, or 100 mg/kg AICAR-treated EAE mice on day 60 after immunization with PLP139–151 peptide. The spleens were pooled and stimulated in vitro with PLP139–151 peptide, and proliferation was measured by [3H]thymidine uptake (a). b and c, The supernatants were collected at 72 h to measure Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokines by ELISA. The CNS infiltrates were detected on day 60 of immunization in EAE (e) and 100 mg/kg AICAR-treated (f) mice and compared between untreated naive SJL/J mice (d). d–f, Fixed and paraffin-embedded spinal cords were sectioned and stained with H&E. Representative sections from five different animals are shown. **, p < 0.01 and ***, p < 0.001 compared to EAE.
infiltration of the inflammatory cells into the CNS. To confirm these results, we used the Hoechst staining method to calculate the degree of infiltration in three different regions of the spinal cord.

We observed a ~2.6-fold increase in infiltration of cells in all the areas (artery, periphery, and center) during EAE disease, whereas treatment with 100 or 500 mg/kg AICAR markedly brought down the infiltration of cells. In fact, a greater degree of reduction was seen in 500 mg/kg AICAR-treated animals (Fig. 2, e (artery), f (periphery), and g (center)).

AICAR induces IL-10 during the late phase of EAE

IL-10 is an immune regulatory cytokine that has been reported to suppress actively induced acute EAE (27–29). Therefore, we aimed to measure IL-10 during a late phase of EAE (day 60) after induction of EAE. On day 60, untreated and AICAR-treated EAE mice were on stage 0 of clinical EAE disease. Spleen cells from these mice were stimulated ex vivo in the presence of PLP139–151 peptide (1–10 μg/ml) to study the recall responses and cytokine profile. As shown in Fig. 3a, the spleen cells of AICAR (100 mg/kg)-treated mice showed greater proliferative responses than untreated mice. Moreover, these cells associated with significantly higher levels of IL-10 (p < 0.001) (Fig. 3b). Of note, there was no difference in the production of TNF-α in treated and untreated
AICAR inhibits Ag recall responses and modulated the Th1- and Th2-type cytokine

The Th1 and Th2 cytokines are key regulators of clinical EAE disease, and their expression is associated with disease severity and attenuation (30). AICAR significantly inhibited \((p < 0.01)\) the recall responses in the spleen cells from PLP\(_{139-151}\)–immunized mice when stimulated ex vivo with PLP\(_{139-151}\) peptide (1–10 \(\mu\)g/ml) in the presence of 0.2 mM AICAR (Fig. 4a). To define whether the anti-inflammatory effects of AICAR on EAE disease associated with alteration in Th1-Th2 profiles, we analyzed the production of Th1 and Th2 representative cytokines from the spleens cells of PLP\(_{139-151}\)–immunized mice after their ex vivo stimulation with peptide in the presence of various concentrations of AICAR (0.1–1.0 mM). The culture supernatants of ex vivo-stimulated cells were examined for Th1 (IFN-\(\gamma\) and TNF-\(\alpha\)) and Th2 (IL-4) cytokines. As shown in Fig. 4, b–d, AICAR reduced the production of Th1 cytokines; however, the production of Th2 cytokine was significantly up-regulated at 200 \(\mu\)M and respective doses \((p < 0.001)\). AICAR also inhibits the Ag-specific recall responses in spleen cells from mice immunized with MOG\(_{35-55}\) after ex vivo stimulation with MOG\(_{35-55}\) peptide in the presence of different concentrations of AICAR (0.1–1.0 mM) (data not shown).

AICAR inhibits the expression of T-bet and induces GATA3 transcription factors

T-bet, GATA3, and STAT6 are the main players in the development of Th1 and Th2 phenotypes, respectively, and regulate the production of IFN-\(\gamma\) and IL-4 in CD4\(^+\) T cells (31, 32). The observed down-regulation of the Th1 cytokine and up-regulation of Th2 cytokine led us to examine the effect of AICAR on their specific transcription factors, T-bet and GATA3, in ex vivo-generated PLP\(_{139-151}\)–specific T cells. AICAR (0.1–1.0 mM) inhibited the expression of T-bet and increased the expression of GATA3 in PLP\(_{139-151}\)–specific T cells (Fig. 5, a and b). In addition, AICAR stabilized the phosphorylation of STAT6 in PLP\(_{139-151}\)–specific T cells (Fig. 5c). Because STAT6 phosphorylation is associated with Th2 cell signaling in EAE disease (33), these results suggest that AICAR profoundly inhibits the Th1-type mediators, whereas it induces or stabilizes the Th2 cell-associated transcription factors.

AICAR-treated M\(\phi\) modulate the PLP\(_{139-151}\)–specific T cells into Th2 lineage cells

The M\(\phi\) were obtained by peritoneal lavage from naïve SJL/J mice 3 days after injection of thioglycolate. The M\(\phi\) were pretreated in vitro with AICAR and cocultured with PLP\(_{139-151}\)–specific T cells in the presence of PLP\(_{139-151}\) (5 \(\mu\)g/ml). The proliferation index of T cells in AICAR-pretreated M\(\phi\) was lower compared with untreated M\(\phi\) (Fig. 6a). Furthermore, AICAR-treated M\(\phi\) skewed the responses to Th2 lineage as evidenced by the increased production of the Th2 cytokines IL-4 and IL-10 \((p < 0.001\) and \(p < 0.05\)) (Fig. 6, b and c). In addition, when PLP\(_{139-151}\)–specific T cells pretreated with AICAR (0.1–1.0 mM) were cocultured with irradiated spleen cells (APCs) pulsed with PLP peptide unable to proliferate (data not shown) indicate direct effect of AICAR on T cells. These results suggest that AICAR was able to act on both APCs and T cells to modulate their function.

AICAR treatment inhibits in vivo expansion of Ag-specific OT-2 CD4\(^+\) T cells

Because EAE is a CD4\(^+\) T cell-mediated autoimmune disease (3), the effect of AICAR was examined on another CD4 T1 model. For this, we used OT-2 TCR transgenic mice, in which CD4 cells recognize OVA\(_{323-339}\) peptide in the context of the MHC-II molecule H-2\(K\)\(^b\). After adoptive transfer to naïve recipients, these CD4 cells expand in vivo and develop Th1-type immune responses after immunization of the host with OVA\(_{323-339}\).
peptide. We used this model to test the impact of AICAR on the in vivo Ag-specific CD4+ T cell expansion. Interestingly, there was 20–35% inhibition in the expansion of OT-2 cells in the PBLs on day 3. The decrease in OT-2 cell expansion persisted until day 5, and the cells were undetectable on day 7 (Fig. 7a). The inhibition of OT-2 expansion by AICAR may reflect the alteration in trafficking of these cells; we therefore analyzed their expansion in DLNs. Substantial inhibition of OT-2 expansion was observed in DLNs of AICAR-treated recipients (Fig. 7b). Fig. 7, c and d, is representative of flow-cytometric data on days 3 and 7 in PBLs and DLNs, respectively. These results confirm the inhibitory effects of AICAR on the Ag-specific responses of CD4+ T cells observed in clinical EAE disease.

Discussion
MS is a chronic inflammatory and autoimmune disease characterized by discrete areas of inflammation and demyelination that can occur in multiple anatomical locations in the CNS and wax and wane in severity over time (1, 4–6). The Th1 CD4+ T cells reactive to myelin produce the proinflammatory cytokines (IFN-γ and TNF-α) that are crucial in orchestrating an immunopathological cascade, mediating the damage to the myelin sheath and eventually the axon (4, 34, 35). In this study, we present data showing AICAR as a novel immunomodulatory agent with beneficial effects on EAE disease. Treatment with AICAR during the course of EAE, in a murine model of MS, induced significant attenuation in the clinical symptoms, as well as in the pathology of disease. By analyzing the mechanism(s) mediating its anti-inflammatory effects, we found that AICAR skewed the immune (inflammatory) response to the anti-inflammatory type through down-regulation and up-regulation of the Th1- and Th2-type immune responses, respectively. Furthermore, AICAR targeted both T cells and APCs and inhibited their functions. Thus, AICAR might be a potential therapy for the MS and other Th1-type-mediated inflammatory diseases.

Administration of AICAR at the time of EAE induction and for 60 days inhibited the clinical disease and pathology in the active EAE, induced by two different myelin peptides, PLP139–151 and MOG35–55, and in the passive disease induced by PLP139–151-sensitized T cells. AICAR is a pharmacological activator of AMPK (8–10), and its role in neurodegeneration and amyloid precursor protein-like/amyloid precursor protein processing has been suggested to be mediated through HMG-CoA reductase (36). Our recent studies show that statins, an HMG-CoA reductase inhibitor, attenuates the disease in the murine disease model and in MS patients (7, 24, 25). Therefore, although the anti-inflammatory effects of statins and AICAR on EAE might be different, their inhibition of HMG-CoA reductase is likely to be a common mechanism. AMPK can also inhibit cholesterol and fatty acid biosynthesis via phosphorylation and inhibition of acetyl CoA-carboxylase and HMG CoA-reductase (9, 11). Previously, it was reported by us and others that AICAR inhibits the production of proinflammatory mediators TNF-α, IL-1β, IL-6, and NO in primary astrocytes, microglia, and Mø (23, 37, 38). Therefore, the observed anti-inflammatory effects of AICAR on EAE are likely mediated through both immune-independent and -dependent mechanisms. Both low (100 mg/kg) and high (500 mg/kg) doses of AICAR profoundly attenuated the severity of the disease in the chronic EAE models induced by PLP139–151 and MOG35–55 peptides in SJL/J and C57BL/6 mice, respectively. Consistent with the decreases in the clinical symptoms, AICAR also decreased infiltration of inflammatory cells into the CNS (Fig. 2). EAE in mice and other rodents is mediated by encephalitogenic CD4+ T cells that produce inflammatory Th1 cytokines such as IFN-γ and TNF-α (39). In addition, cytokine-induced immune deviation has been investigated as potential therapy for Th1-mediated autoimmune diseases, because cytokines present at the time of activation may alter the pathogenicity of effector T cells (40). Thus, one possible mechanism for the immune-mediated anti-inflammatory effects of AICAR on EAE is modulation of Th1 and Th2 responses. By analyzing the quantity and quality of the immune responses in EAE mice, we found that AICAR impaired Ag-specific recall responses (Fig. 4a), coinciding with inhibition of the proinflammatory Th1-type cytokines IFN-γ and TNF-α (Fig. 4, b and c) and the Th1-associated transcription factor T-bet (Fig. 5a). In contrast, AICAR induced significant levels of the anti-inflammatory Th2 cytokine IL-4 (Fig. 4d), induced expression of the Th2-associated transcription factor GATA3, and induced phosphorylation of STAT6 (Fig. 5, b and c). In addition to its roles in the generation of Th2-type response, STAT6 has been shown to play significant roles in EAE disease in which STAT6-deficient mice have a more severe EAE compared with the wild-type mice (33).

Our data show that AICAR not only down-regulates the Th1-type responses in EAE mice but also up-regulates the Th2 response. Although our data do not explain whether the effects of AICAR on the Th1- and Th2-type responses are selective or secondary effects to modulation of either response, the combined effects of AICAR on Th1/Th2 responses are likely to be a working mechanism mediating its immunomodulatory effect on EAE disease. The anti-inflammatory effects of AICAR were also confirmed in an irrelevant model in which treatment of host mice receiving transgenic OT-2 CD4+ T cells with subsequent peptide immunization suppressed the generated T cell responses (Fig. 7).

Because AICAR is a potent activator of AMPK (9, 10) and activation of AMPK was found to negatively regulate the activation of IkB kinase α and NF-κB, a required event in the immune responses (23). Hence, attenuation of EAE by AICAR highlights the regulatory role of AMPK in the inflammatory diseases. Although the role of AMPK in modulation of the Th1/Th2 axis has not been addressed, biasing the T cell response in EAE mice to Th1/Th2 after AICAR treatment indicates that the AMPK system might play an important role in the differentiation of Th1/Th2 axis. The differential effects of AICAR on the Th1- and Th2-type responses could be due to its effect on T cells and/or APCs. The effect of AICAR on APCs (microglia and astrocytes) is dependent on AMPK (23), but modulation of other APCs and T cells by AICAR is yet to be defined. Our data show that AICAR is likely to modulate both APCs and T cells. Specifically, treatment of Ag-pulsed APCs (peritoneal Mø) with AICAR impaired their Ag presenting capability when cocultured with PLP139–151-sensitized T cells. However, in vitro these AICAR-pretreated, peritoneal Mø were able to increase the production of Th2-type cytokines (IL-4 and IL-10) when cocultured with PLP139–151-specific T cells (Fig. 6, b and c). Studies are underway to investigate how AICAR affects the functions of APCs or T cells to produce these immune regulatory cytokines. Similarly, treatment of PLP139–151-specific T cells with AICAR inhibited their Ag-specific proliferation in response to restimulation in vitro with irradiated APCs loaded with PLP139–151 peptide (data not shown). Collectively, these data suggest that AICAR impairs the APC-T cell functions resulting in inhibition of the Th1-type responses. The latter effect might permit generation of the Th2-type response, amplifying the anti-inflammatory effects of AICAR.

We observed more proliferation of T cells in AICAR-treated cells with increased IL-10 production (Fig. 3, a and b). Based on our ex vivo APC and T cell coculture (Fig. 6), we suggest that AICAR affects both T cells and APCs during the EAE disease process. The proliferation of T cells from AICAR-treated mice was not inhibited, indicating that AICAR treatment did not interfere...
with the proliferation of spleen cells during ex vivo recall responses; however, it did alter the nature of responding cells toward immune regulatory cells, driving them to produce more IL-10.

The up-regulation of the Th2-type response by AICAR treatment was not a transient effect, in which we found an induction of IL-10 from spleen cells after ex vivo stimulation on day 60 of EAE. At the same time point, infiltration of inflammatory cells into the CNS was greatly reduced in the AICAR-treated EAE mice compared with control EAE mice (Fig. 3, e and f). IL-10 is an immunoregulatory cytokine crucial in inflammatory reactions with potent anti-inflammatory and immunosuppressive activities (41). IL-10 has been used in the treatment of many disease models, augmenting humoral immune responses and inhibiting certain aspects of cellular immunity (28, 40, 41). IL-10 is associated with disease remission in EAE and MS, in which its levels were found to be high in IFN-β-treated patients (42). IL-10 mRNA was seen to be regulated during the remission phase of EAE (43), and IL-10-deficient mice fail to recover from MOG-induced EAE, whereas mice transgenic for IL-10 are completely resistant to the development of EAE (28). In addition, mucosal (nasal) administration of IL-10 has been shown to suppress Th1 responses and enhance production of TGF-β/IL-10 when given with an auto-antigen, in EAE (27). Interestingly, we observed that although AICAR at 100 mg/kg had relatively less effect on the infiltration of immune cells into the CNS at the first peak of the disease, it significantly reduced the cell infiltration on day 60. This suggests low doses of AICAR may need a longer time to up-regulate the immune regulatory process, which could reduce the generation of inflammatory cells and their infiltration in the CNS. Through analysis of spinal cord pathology on day 60, we observed a significant decrease in the number of infiltrating cells in AICAR-treated EAE animals. However, at this stage, treated and untreated animals had no clinical disease. This may reflect that some infiltrates are required for the observed disease phenotype or that properties of the inflammatory cells in the spinal cord are different from those found in the CNS during the active phase of the disease.

Although our findings show immune-mediated mechanisms for the anti-inflammatory effects of AICAR on EAE, we cannot exclude other effects. For instance, AICAR has been shown to inhibit cholesterol biosynthesis; however, their mechanism of action is different, because the addition of mevalonate is unable to reverse the effect of AICAR (23). AMPK has been reported to have a different, because the addition of mevalonate is unable to reverse the effect of AICAR (23).

Potent anti-inflammatory effects of AICAR on EAE, we cannot exclude other effects. For instance, AICAR may need a longer time to up-regulate the immune regulatory process, which could reduce the generation of inflammatory cells and their infiltration in the CNS. Through analysis of spinal cord pathology on day 60, we observed a significant decrease in the number of infiltrating cells in AICAR-treated EAE animals. However, at this stage, treated and untreated animals had no clinical disease. This may reflect that some infiltrates are required for the observed disease phenotype or that properties of the inflammatory cells in the spinal cord are different from those found in the CNS during the active phase of the disease.

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Disclosures

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References


