Inhibition of Transmethylation Down-Regulates CD4 T Cell Activation and Curtails Development of Autoimmunity in a Model System

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*J Immunol* 2007; 178:5366-5374; doi: 10.4049/jimmunol.178.8.5366

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Inhibition of Transmethylation Down-Regulates CD4 T Cell Activation and Curtails Development of Autoimmunity in a Model System

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Transmethylation affects several cellular events, including T cell activation, and blockade of this pathway may curtail inflammatory/autoimmune responses. Here, we demonstrate that transmethylation inhibition by a novel reversible S-adenosyl-l-homocysteine hydrolase inhibitor leads to immunosuppression by reducing phosphorylation of several key proteins involved in TCR signaling, including Akt, Erk1/2, and NF-κB. Remarkably, this effect was largely restricted to CD4 T cells and correlated with reduced arginine methylation of Vav1, an essential guanine nucleotide exchange factor in T cell stimulation. Treatment with the transmethylation inhibitor averted, and even ameliorated, the CD4-mediated autoimmune disease, experimental autoimmune encephalomyelitis. The data suggest that transmethylation is required for CD4 T cell activation, and its inhibition may be a novel approach in the treatment of multiple sclerosis, and other CD4-mediated autoimmune diseases. The Journal of Immunology, 2007, 178: 5366–5374.

Nucleic acid sequences rarely predict the final molecular state and activity of the encoded proteins, because post-translational modifications frequently contribute essential functional characteristics (1). Although phosphorylation has long been studied as the major posttranslational modification, methylation, and in particular arginine methylation, has recently begun to attract attention and has been shown to affect many cellular processes by modifying not only proteins but also nucleic acids and phospholipids (2–4). The major transmethylation pathway involves methyltransferase-mediated donation of methyl groups by S-adenosylmethionine and conversion of S-adenosylmethionine to S-adenosyl-L-homocysteine (SAH), a potent feed-back inhibitor of methyltransferases (5). This reaction is tightly regulated by S-adenosyl-L-homocysteine hydrolase (SAHase), a highly conserved ubiquitous enzyme that catalyzes the hydrolysis of SAH into adenosine and homocysteine.

Transmethylation is particularly important for the normal function of the immune system. For example, blockade of SAHase was associated with decreased Ig production and slower cell growth in a lymphoblastoid cell line (6) and decreased humoral and cell-mediated immune responses (7–10). Mice deficient in the arginine methyltransferase CARM1 showed a partial developmental arrest in an early thymocyte progenitor subset (11). A critical role of transmethylation in the normal development and function of the immune system is further suggested by the human disease adenosine deaminase deficiency, in which the inability to convert adenosine to inosine is associated with an ~50% reduction in SAHase activity (6). Lymphocytes appear to have a higher requirement for transmethylation reactions than other cell types, particularly when activated (12).

Initial findings of immunosuppression by transmethylation inhibition provided the impetus to identify clinically useful compounds to treat autoimmune/inflammatory diseases. A major hurdle in this effort was the irreversible action of such compounds and associated severe toxicity (5). Recently, however, we developed a potent, but reversible, SAHase-inhibiting compound (methyl 4-(adenin-9-yl)-2-hydroxybutanoate (DZ2002) and demonstrated its immunosuppressive activity (7, 8). This compound was rationally designed based on the x-ray crystal structure of SAHase (13), and computational binding studies which compared an irreversible SAHase inhibitor that reduced enzyme-bound NAD⁺ to NADH with a reversible SAHase inhibitor (D-Eritadenine) that did not reduce NAD⁺ to NADH or alter the structure of SAHase. DZ2002 was chosen from a panel of D-Eritadenine derivatives because of similar inhibitory activity (Kᵢ, 17 nM) (7), but with a larger kᵢₒ value (higher rate of dissociation) that renders it more reversible and less toxic.

The availability of this compound prompted us to further examine the mechanisms by which transmethylation inhibition induces immunosuppression and the potential therapeutic efficacy of this approach in a prototypic model of autoimmune disease, experimental autoimmune encephalomyelitis (EAE). This disease, primarily mediated by CD4 Th1 cells, can be induced in animals by the administration of myelin basic protein or other myelin protein derivatives, such as proteolipid protein (PLP₁₃₀–₁₅₁) and myelin oligodendrocyte glycoprotein (MOG) and is considered a useful model for multiple sclerosis (14–18).

Here, we document that transmethylation inhibition interferes with both proximal and distal events in TCR signaling primarily...
lected with G418. Knockdown of SAHase (PCR (21). Briefly, RNA was isolated from several control and 0.4% DMSO in PBS) for 2 h and then stimulated with magnetic beads for gel loading, cleared lysates were analyzed for and digitized using an Epson Perfection 4870 PHOTO scanner. To control noprecipitated with anti-mono and dimethyl arginine (DMA) Abs (Abcam), and amide gels (20 times. Cells were allowed to rest for an additional 2 h; then cells were incubated with 100 μM DZ2002 or vehicle (0.4% DMSO in PBS) for 2 h and then stimulated with magnetic beads (Miltenyi Biotec) loaded with anti-CD2-, -CD3, and -CD28 (all Abs at 100 μg/ml) for 1 h. In preliminary experiments, treatment with DZ2002 at concentrations up to 1 mL did not cause either apoptosis or necrosis in primary cultured T cells or cell lines for up to 24 h (data not shown). Protein was extracted in the presence of protease and phosphatase inhibitors (Pierce Biotechnology), and quantitated by the Bradford assay (Bio-Rad). Proteins were then separated on 4–12% SDS-PAGE polyacrylamide gels (20 μg/lane), transferred onto polyvinylidene difluoride membranes, blocked with nonfat milk powder, and probed with Abs to total and then phosphorylated Akt, Etk1/2, NF-κB, Lck, STAT1, and STAT3 (all from Cell Signaling Technology). After membranes were initially probed with Abs to total protein levels, most membranes were stripped and re-probed for anti-phosphorylated protein levels, although in a few instances separate gels were run. Bands were revealed with secondary anti-rabbit or anti-mouse IgG-HRP Abs (BioLegend) as appropriate and developed for chemiluminescence. Membranes were then stripped and reprobed with anti-β-actin Abs (BioLegend) to confirm equal protein loading.

For Vav1 methylation assessment, cleaved protein lysates were immunoprecipitated with anti-mono and dimethyl arginine (DMA) Abs (Abcam), and methylated proteins were isolated with protein G. Washed beads were separated on an 8–16% SDS-PAGE gel and immunoblotted with anti-Vav1 Ab (Santa Cruz Biosciences; Ref. 19). Proteins were visualized by Super Signal West-Femto Chemiluminescent Substrate (Pierce Biotechnology), and digitized using an Epson Perfection 4870U PHOTO scanner. To control for gel loading, cleared lysates were analyzed for β-actin (Fig. 2) as well as total Vav1 protein levels, which were equal (data not shown).

Calcium flux
Mouse splenocytes were pretreated with either 100 μM DZ2002 or vehicle for 2 h at 37°C. Cells were then loaded with Indo-1, acetoxyethyl ester (2 μg/ml) dissolved in pluronic-127 in the presence of probenecid (4 mM), all from Invitrogen, for 30 min at room temperature in the dark and labeled with CD4-PE, CD8-allophycocyanin, and biotinylated anti-CD3 and -CD28 (all from BioLegend). Each sample was warmed to 37°C before analysis. Baseline fluorescence was monitored for ~2 min before addition of avidin (60 μg/ml; Vector Laboratories) to cross-link the biotinylated anti-CD3/CD28 Abs (20). Calcium flux data were acquired for 10–15 min at 351 nm at room temperature from 0 to 7 min on a UV-1601PC spectrophotometer (Shimadzu; Ref. 7). K_i was calculated by the equation K_i = K_m(1 + [substrate]/K_m) in which K_m = [inhibitor]/(V/Vo) – 1, and K_m = 7.9 μM.

Additionally, SAHase (0.5 mg/ml) was incubated with DZ2002 (100 μM) in 50 mM sodium phosphate buffer, pH 7.2, for 10 min at room temperature. SAHase and the SAHase-DZ2002 mixture were dialyzed against 50 mM sodium phosphate buffer, pH 7.2, for 24 h and the activity of SAHase was measured as above, both before and after dialysis.

Mice
Female SJL mice (4–6 wk old) were purchased from The Jackson Laboratory. Animals were housed in specific pathogen-free conditions, and all procedures were approved by The Torrey Pines Institute for Molecular Studies’ Animal Research Committee (La Jolla, CA).

EAE induction
Myelin PLP139–151 (HSLGTKWLGHPDKF) was synthesized at NeoMPS, and 4 mg/ml of this peptide in PBS were emulsified 1:1 in IFA (Difco) supplemented with 4 mg/ml of lipopolysaccharide (E. coli 055:B5; Difco); 100 μl of emulsion containing 200 μg of PLP139–151 were injected s.c. into three different spots on the backs of female SJL mice (10 mice/group). Additionally, 200 ng of pertussis toxin (PTX) in PBS was administered i.p. on days 0 and 2 (22). Mice were monitored daily, and paralysis was scored blindly according to the following criteria: 0, no observable disease; 1, limp tail; 2, limp tail and partial weakness; 3, one hind limb paralyzed; 4, both hind limbs paralyzed; and 5, moribund or dead (23).

Adaptive transfer of EAE
Donor female SJL mice (n = 10) were immunized with 200 μg of PLP139–151 in CFA as above, and reimmunized on day 7 with PLP139–151 in IFA (Difco). Spleens were harvested on day 10, RBCs were lysed, and cells (5 × 10^6 cells/ml) were cultured in RPMI-10% FBS, supplemented with 30 μg/ml PLP139–151, and 10 ng/ml recombinant mouse IL-12 (BioLegend). After 96 h, cells were washed, resuspended in PBS, and injected i.p. (7.2 × 10^6 cells/mouse) into 6- to 8-wk-old female SJL naive recipient mice (n = 10/mice/treatment group; Ref. 22). PTX was administered to these recipients on days 0 and 2, and disease severity was scored as indicated above.

Treatment protocols
The SAHase inhibitor DZ2002 was synthesized at Diazyme Laboratories, dissolved in vehicle, and delivered either i.p. (50 mg/kg/day) or s.c. by implanted osmotic pump (Alzet) at 0, 0.1, 1, and 10 mg/kg/day. Osmotic pump DZ2002 concentration was calculated on the basis of osmotic pump daily release rate and total mouse body weight. Prophylactic treatment (osmotic pump delivery of DZ2002) was initiated simultaneously with the initial PLP139–151 immunization, whereas therapeutic treatment (i.p. delivery of DZ2002) began only after clinical symptoms began to appear (~day 11 post-PLP139–151 challenge; disease score, ≥1). The dosage of DZ2002 was determined by a preliminary acute toxicity study, which indicated that the LD50 of DZ2002 administered i.p. was ~520 mg/kg/day (data not shown). We administered 10 times less than the LD50 and, indeed, observed no signs of overt toxicity in any mice over the course of these studies. Additionally, mice treated for ~9 mo with daily i.p. injections of DZ2002 (50 mg/kg/day) also showed no signs of toxicity (data not shown).

To confirm that the mechanism of immunosuppression was inhibition of SAHase, a DZ2002 analog lacking the SAHase-inhibitory activity of DZ2002 was also synthesized, and administered at 10 mg/hind limb by osmotic pump.

Histology
DZ2002- and vehicle-treated mice were sacrificed on day 13 (the peak of disease in the control group) and perfused with PBS, and spinal cords were extracted and fixed in 10% buffered formalin. Paraffin-embedded, 4-μm thick sections of mouse spinal cord were Luxol-fast blue stained to determine immune cell infiltration and degree of demyelination. Complete histological examination was also performed.

In vitro SAHase inhibition assay
The SAHase inhibitor or its inactive analog was dissolved in vehicle. The assay reaction contained 50 mM phosphate buffer (pH 7.4), 320 μM SAH, 100 μM 5′,5′-dithiobis(2-nitrobenzoic acid), 50 μM SAHase, and 0.5 μM DZ2002, 0.5 μM analog, or vehicle. Optical density was recorded at 405 nm at room temperature from 0 to 7 min on a UV-1601PC spectrophotometer (Shimadzu; Ref. 7). K_i was calculated by the equation K_i = K_m(1 + [substrate]/K_m) in which K_m = [inhibitor]/(V/Vo) – 1, and K_m = 7.9 μM.
Splenocytes were harvested on day 13 post-PLP139–151 challenge, and RBCs were lysed in ammonium chloride solution and stained with Abs to CD4, CD8, CD19, CD25, CD29 (VLA-4/H9252-chain), CD40, CD40L, CD44, CD49d (VLA-4/H9251-chain), CD69, CD80, CD86 (all from BioLegend), or F4/80 (Caltag). For intracellular cytokine analysis, splenocytes were stimulated with PLP (10 μg/ml) for 48 h, treated with brefeldin A (3 μg/ml), and stained with Abs to CD4, CD8, or CD19. After fixation and permeabilization, cells were stained with allophycocyanin-conjugated Abs to IL-2, IL-4, IL-10, IFN-γ, or TNF-α (all from BioLegend). Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Acute and recall responses to PLP139–151

For acute EAE responses, disease was induced, and mice were treated with either DZ2002 or vehicle for 13 days (10 mice/treatment group). For recall EAE responses, disease was induced, and mice were treated with either DZ2002 or vehicle for 42 days (10 mice/treatment group). At the indicated times, spleens were harvested, and cells were stimulated in vitro on microtiter plates (2 × 10^5 cells/well) with PLP139–151 (10 μg/ml) for 72 h at which time WST-8 reagent was added according to the manufacturer’s instructions (Dojindo). OD was read at 450 nm. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product. The amount of formazan produced is directly proportional to the number of living cells, and the detection sensitivity of cell proliferation using WST-8 was shown to be equivalent to the thymidine incorporation assay.

Similar cultures were set, and supernatants were collected at 48 h and assessed by ELISA for the presence of IL-2, IL-4, IL-10, IFN-γ, and TNF-α using commercially available Ab pairs (BioLegend).

Statistics

EAE disease severity scores are depicted as the average of the treatment group per day over the treatment period and were analyzed using the Kruskal-Wallis test. All other group mean comparisons were conducted using Student’s t test. Statistical analyses were performed using MedCalc for Windows, version 8.1.0.0 (MedCalc Software). p < 0.05 was considered significant.

Results

Inhibition of SAHase reduces TCR signaling

Immunosuppression mediated by transmethylation inhibition may be due to interference with proximal and/or distal events associated with TCR signaling. To examine this possibility, we cultured total splenocytes from SJL mice in the presence or absence of the reversible SAHase inhibitor, DZ2002 for 2 h and then stimulated these cells with anti-CD2-, -CD3-, or -CD28-coated beads. Protein lysates were prepared at intervals for up to 1 h and assessed for levels of relevant phosphoproteins. We observed significant reductions in phosphorylated Akt and Erk1/2, whereas phosphorylated NF-κB, Lck, STAT1, and STAT3 were unaffected (Fig. 1, A and B).
Signaling defects caused by SAHase inhibition are restricted to the CD4 subset

To evaluate whether decreases in TCR signaling by transmethylation inhibition were T cell lineage specific, we repeated the above experiments with purified (>95%) CD4 and CD8 T cells. Strikingly, substantial reductions in phosphorylated Akt (~16.1-fold), Erk1/2 (~8.4-fold), and NF-κB (~2.1-fold) were restricted to the DZ2002-treated CD4 subset, whereas the DZ2002-treated CD8 T cells showed increases in these phosphoproteins (Fig. 1, C and D).

Inhibition of transmethylation reduces calcium mobilization by activated CD4 T cells

Reductions in TCR signaling induced by transmethylation inhibition will likely be reflected by the intensity of calcium mobilization. To assess calcium flux, primary T cells were pretreated for 2 h with either vehicle or DZ2002 and activated with anti-CD3 and -CD28, and the intensity of calcium mobilization was measured. Calcium mobilization was significantly reduced in DZ2002-treated CD4 T cells with the area under the induction curve being 44.5% less than the control cells (Fig. 1E). Contrastingly, the reduction in calcium flux of DZ2002-treated CD8 T cells was more modest (16.2%).

Vav1 methylation is SAHase dependent

It has been demonstrated that CD28 engagement induces protein arginine methyltransferase activity and the subsequent methylation of arginine on Vav1 (19). An irreversible inhibitor of SAHase reduced methylation of Vav1, resulting in decreased IL-2 production. Consistent with these findings, we found that a 2-h pre-treatment with DZ2002 caused a decrease in Vav1 methylation of both naive mouse CD4 T cells and Jurkat cells. After activation with anti-CD3 and -CD28, this decline became even more evident at 30 and 60 min (Fig. 2A). Vav1 methylation of CD8 T cells appeared mostly unaffected by DZ2002 up to 30 min. Decreases in Vav1 methylation at 30 min were confined to the CD4 subset (Fig. 2B).

SAHase knockdown impairs T cell activation

To document the reversibility of DZ2002, we cultured Jurkat T cells in the presence or absence of DZ2002 for 2 h, removed the compound and, after allowing cells to equilibrate for 2 more hours, stimulated these cells and assayed for levels of phosphorylated Akt. Once the SAHase inhibitor was removed, TCR signaling was restored in the DZ2002 washout cultures (Fig. 4A). As a confirmation of these results, we also incubated DZ2002 with SAHase, removed the compound by overnight dialysis, and showed that SAHase activity was almost fully recovered (Fig. 4B).

Acute EAE is reduced by transmethylation inhibition

The results clearly indicate that the reversible transmethylation inhibitor DZ2002 potently suppresses T cell activation in a CD4-specific manner. Because EAE is mediated by myelin-recognizing CD4 T cells (14), we examined whether inhibition of transmethylation reactions could result in blockade and/or amelioration of this disease. EAE was induced by immunizing SJL/J mice with PLP139–151 emulsified in CFA followed by PTX (22). A continuous supply of the inhibitor was ensured by s.c. implantation of osmotic pumps 3 days before EAE induction. Blockade of SAHase by DZ2002 (0.1–10 mg/kg/day) resulted in a significant dose-dependent reduction of EAE up to day 38, the last time of observation. Optimum effects were observed with doses of 10 (Fig. 5A) or 1 mg/kg/day, but 0.1 mg/kg/day only had a modest effect (Fig. 5C). At day 14 (the peak of disease), DZ2002-treated mice showed an average disease severity score of ~1.4 compared with
~3.2 in the vehicle-treated controls. Moreover, the mean area under the disease severity curves showed an ~2.3-fold overall reduction in the DZ2002-treated group, with ~70% of the DZ2002-treated mice vs only ~30% of the controls being disease-free by day 25 (Fig. 5D). Mice receiving the highest DZ2002 dose also showed an ~3.1-fold decrease in infiltrating cells and a substantial reduction in demyelination compared with controls (Fig. 5E). The frequencies of total T or B cells in the spleens of treated animals were unaltered; however, there was a significant reduction in CD4, but not CD8, T cells expressing activation/memory markers (CD44high and CD69) or integrin β1 (Table I). Moreover, disease reduction by DZ2002 was due to SAHase inhibition because mice treated with an analog lacking a hydroxyl group and devoid of SAHase-inhibitory activity (Fig. 6A) was ineffective (Fig. 6B).

**Reduction of SAHase ameliorates established EAE**

Encouraged by the efficacy of DZ2002 in the treatment of acute EAE, we assessed whether this compound could also reduce established disease. EAE was induced, and at the first sign of disease (score, ≥1), mice were randomly assigned to either DZ2002- or vehicle-treated groups. In contrast to the preventive treatment protocol, DZ2002 (50 mg/kg/day) and vehicle were administered by i.p. injection once daily. This dose and injection schedule was based on previous observations that SAHase levels in liver were <50% of normal up to 24 h after administration of an SAHase inhibitor (5) and that such a regimen given for ~9 mo had no discernable adverse effects (data not shown). By day 16 (peak of disease), both treatment groups showed similar severity scores (Fig. 7A). Strikingly, however, DZ2002-treated mice rapidly recovered, and by day 25 most of these mice were disease free with a mean score of ~0.3 out to day 42. These data are in stark contrast to control animals that showed an overall incidence of ~85% and a mean score of ~1.5 at this time point (Fig. 7B). Moreover, comparison of the mean areas under the curves depicting disease severity showed an ~2.4-fold reduction in DZ2002-treated mice compared with controls.

To determine whether disease would relapse after SAHase activity had been restored, DZ2002 treatment was discontinued from the therapeutically treated animals on day 45 and mice were followed for disease recurrence. A gradual increase of disease was observed, and by day 65 incidence and severity were indistinguishable between the groups that had previously been treated with vehicle or DZ2002 (severity scores, ~1.3 vs ~1.2, respectively). The results suggest that SAHase inhibition does not eliminate autoreactive T cells but rather temporarily suppresses them, and effector function is resumed with restoration of SAHase activity.

**Inhibition of SAHase suppresses pathogenic T cells**

EAE is transferable by encephalitogenic CD4 T cells (22). We used adoptive transfer experiments to determine whether SAHase blockade reduces the ability of pathogenic T cells to induce disease. Donor SJL mice were immunized with PLP131–159 in CFA, and 10 days later splenocytes were restimulated in vitro with PLP130–151 and IL-12. The resulting primed T cells (>95% CD3+ were injected (7 × 10⁶) into naive syngeneic recipients together with PTX on days 0 and 2. These mice were then treated or not with DZ2002 (50 mg/kg/day i.p.). Compared with controls,
DZ2002-treated mice showed marked decreases in disease incidence (data not shown), onset, and severity, as indicated by an ~2.6-fold reduction in the mean area under the curves depicting EAE scores (Fig. 7C). These results indicate that autoreactive T cells constitute a primary target for SAHase inhibition resulting in disease reduction.

SAHase inhibition suppresses proliferative and cytokine responses to PLP<sub>139-151</sub>

To further document the effects of transmethylation inhibition on autoreactive T cells, we examined in vitro T cell proliferation and cytokine production in response to PLP<sub>139-151</sub> from splenocytes derived from mice at either the acute (day 13) or recall (day 42) phases of EAE. Compared with controls, DZ2002-treated mice at the acute phase showed a 70% reduction (Fig. 8A, left), and those at the recall phase a 50% reduction (Fig. 8B, left) in proliferative responses. Th1-type cytokines, thought to be involved in the pathogenesis of EAE (14), were also significantly reduced in DZ2002-treated cells at the acute phase, as indicated by both intracellular staining (Table I) and levels in supernatants (Fig. 8A, right). Thus, IL-2 (101.6 ± 17.6 vs 35.7 ± 3.8 pg/ml), IFN-γ (1,780.2 ± 78.4 vs 696.3 ± 40.9 pg/ml), TNF-α (725.6 ± 89.4 vs 217.3 ± 16 pg/ml), and IL-12p40 (1,181 ± 72.7 vs 540.9 ± 89.4 pg/ml) were reduced, whereas IL-4 was somewhat increased (9.4 ± 2.1 vs >4 pg/ml). Because the IL-12p40 subunit is shared by IL-12 and IL-23, this reduction may be due to effects on either or both of these cytokines. Similar reductions in inflammatory cytokines, i.e., IL-2 (38.0 ± 3.7 vs 82.8 ± 8 pg/ml), IL-12p40 (94.6 ± 9 vs 192.8 ± 19 pg/ml), and TNF-α (38.8 ± 3.5 vs 116 ± 11 pg/ml), albeit at lower levels, were also noted at the recall phase.

Table I. T and B cell frequency in DZ2002-treated EAE mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Cell Populations</th>
<th>Control (%)</th>
<th>DZ2002 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>28.3 ± 3.2</td>
<td>31.5 ± 5.3</td>
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<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;+&lt;/sup&gt;</td>
<td>12.8 ± 0.8</td>
<td>11.8 ± 0.7</td>
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<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;+&lt;/sup&gt;</td>
<td>36.3 ± 0.05</td>
<td>31.1 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.4 ± 2.3</td>
<td>33.4 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD69&lt;sup&gt;+&lt;/sup&gt;</td>
<td>15.4 ± 1.3</td>
<td>11.8 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>9.0 ± 1.1</td>
<td>7.0 ± 0.8</td>
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<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>29.8 ± 3.4</td>
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<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.7 ± 3.4</td>
<td>26.5 ± 4.8</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;CD69&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.8 ± 0.5</td>
<td>2.9 ± 0.2</td>
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<tr>
<td>CD19&lt;sup&gt;+&lt;/sup&gt;</td>
<td>38.6 ± 1.4</td>
<td>41 ± 0.5</td>
</tr>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD19&lt;sup&gt;+&lt;/sup&gt;</td>
<td>31.7 ± 2.7</td>
<td>13.0 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD69&lt;sup&gt;+&lt;/sup&gt;</td>
<td>29.3 ± 3.9</td>
<td>12.3 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;IL-4&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;IFN-γ&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30.0 ± 3.5</td>
<td>17.7 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>EAE was induced in SJL mice, and T and B cell frequency was determined on day 14. Additionally, splenocytes were cultured for 48 h with PLP<sub>139-151</sub> (10 μg/ml) and intracellular cytokine production was determined by FACS. Numbers indicate percentages of total splenocytes or the indicated subset ± SEM.

<sup>b</sup>p < 0.05 between control and DZ2002-treated animals (8 mice/group). Data are representative of two independent experiments.

FIGURE 6. DZ2002 is specific for SAHase. <i>A</i>, In vitro SAHase inhibition assay. Control (——), DZ2002 (- - -) or inactive analog (Δ). <i>B</i>, Group mean disease severity scores in vehicle (●)- and analog (△)-treated mice (10 mice/group). Data are representative of two independent experiments.

FIGURE 7. DZ2002 ameliorates both established and adoptively transferred EAE. <i>A</i>, Group mean disease severity scores in vehicle (●)- and DZ2002 (○)-therapeutically treated mice (10 mice/group). DZ2002 was not administered until the animals began showing signs of disease (~day 11; score, ≥1); p = 0.0024. <i>B</i>, Percentage of mice in either group from A showing a score of ≥1. <i>C</i>, Group mean disease severity scores in vehicle (●)- and DZ2002 (○)-treated mice (10 mice/group) after transfer of encephalitogenic T cells; p < 0.0005. Data are representative of four independent experiments.
in the DZ2002-treated cells, but IL-4 and IFN-γ were undetectable in both treated and control cells at this phase (Fig. 8B, right).

Discussion

On the basis of the results obtained using a novel small molecule inhibitor with potent but reversible activity, we propose several mechanisms by which inhibition of transmethylation leads to immunosuppression. We demonstrate that inhibition of transmethylation results in reductions in both proximal and distal TCR signaling events, including methylation of Vav1 and phosphorylation of Akt, Erk1/2, and NF-κB. Remarkably, transmethylation inhibition selectively affected the above TCR signaling events in CD4, but not CD8, T cells, suggesting that these two lineages have differing methylation requirements for activation. Consequently, we applied this inhibitor to EAE, a CD4-mediated autoimmune disease, and demonstrated its efficacy in blocking disease development and, more importantly, ameliorating established disease. The reversibility of the effects of DZ2002 and rapid restoration of SAHase enzyme activity after discontinuation of treatment with this compound are characteristics that confer potential utility of this approach to the treatment of human autoimmune syndromes.

Initially, the potential therapeutic utility of transmethylation inhibition focused on viral diseases (24, 25). More recently, however, applications to other clinical entities such as cardiovascular diseases, cancer, and autoimmunity have been considered (5, 7, 8, 26). Inhibition of transmethylation reactions may be accomplished either directly by blockade of methyltransferases, or indirectly by reducing SAHase activity. To date, however, designing specific methyltransferase inhibitors of clinical utility has been unsuccessful. Moreover, early classes of SAHase blockers acted irreversibly by either permanent trapping of the oxidized inhibitor onto the enzyme (type I), or by covalent modification of the active site of the enzyme (type II) (5). Although these compounds exhibited potent antiviral and immunosuppressive effects, the delay in reestablishing normal levels of SAHase (~5 days; data not shown) led to severe toxicity, thereby precluding their clinical use. In fact, deletion of a segment on chromosome 2 in mice containing the Ahcy gene led to severe developmental defects that were reproduced in embryos cultured in the presence of an irreversible SAHase inhibitor or metabolites that elevate SAH (27). Therefore, efforts were made to develop reversible inhibitors of transmethylation, and among them DZ2002 was shown here, and previously, not to cause any overt toxicity, likely due to only ~50% reductions in SAHase levels (8). In this regard, even in the case of more severe reductions in SAHase activity in humans due to genetic mutations, the associated metabolic defects were correctable by dietary restrictions and/or supplements (28).

Our in vitro studies showed that T cell signaling events were significantly reduced in DZ2002-treated T cells, including phosphorylation of Akt, Erk1/2, and NF-κB and calcium mobilization. These effects appear to result from interference with Vav1 methylation, a guanine nucleotide exchange factor critical in the transduction of TCR signals (29). Others have also shown that Vav1 methylation accompanies CD28-costimulatory signaling and that this methylation allows nuclear translocation of Vav1 and subsequent increases in IL-2 production (19). In this report, however,
superantigen-induced global tyrosine phosphorylation was shown to be unaltered by treatment with an irreversible SAHase inhibitor. The differences with our findings may primarily be attributed to the fact that Blanchet et al. assessed this parameter with total T cells, whereas we examined CD4 cells shown here to be the primary T cell target of transmethylation inhibition. Other, less likely possibilities include differences in the activation stimulus (superantigen vs anti-CD3/CD28) and the concentration of SAHase inhibitors (1 vs 100 μM).

Reductions in the proximal and distal events in TCR-signaling pathways were almost exclusively confined to the DZ2002-treated CD4 T cell subset. This finding supports the recent studies of Prisco et al. (30) in which mice carrying an arginine to glycine point mutation in the pleckstrin homology-domain of Vav1 showed selective CD4+, but not CD8+, perturbations in TCR-induced downstream activation pathways, despite the requirement of Vav1 in both lineages. No clear explanation for this lineage-specific effect of the pleckstrin homology domain mutation was provided, but the present findings suggest that differences in arginine methylation of this domain between CD4 and CD8 cells is the determining factor. In accordance with the above report, we suggest that Vav1 must be properly methylated before TCR activation; and in the absence of this posttranslational modification, TCR signaling through Vav1 is inhibited, at least in CD4 T cells.

The finding that transmethylation inhibition curtailed the effector function of CD4 T cells, and the previously noted higher methylation requirements in activated than resting T cells (9, 12), constituted the basis for assessing the effectiveness of DZ2002 in EAE, a model CD4-mediated autoimmune disease (14–16). DZ2002 was highly effective in reducing incidence, onset, and severity of not only acute but also established EAE, results highly compatible with our separate studies using MOG<sub>35–55</sub>-immunized mice (31) and those of Moreno et al. (32), using a separate SAHase inhibitor, methylthioadenosine, in a rat model of EAE. In our MOG-induced model, we also observed a reduction in proliferation of DZ2002-treated T cells associated with the down-regulation of several cyclin-dependent kinases and up-regulation of a pancyclin inhibitor. The study using methylthioadenosine demonstrated reduced T cell proliferation, which was ascribed to a blockade in T cell signaling likely due to the prevention of IskB-α degradation and impaired activation of the NF-xB pathway. Overall, these findings are in agreement with our current study, which further documents that SAHase inhibition causes deficits in TCR signaling by primarily affecting early signaling events dependent on Vav1 activation, particularly in the CD4 T cell subset.

These effects were clearly mediated by transmethylation inhibition, because a closely related analog devoid of SAHase-blocking activity was ineffective. These beneficial effects were associated with reductions in CNS infiltrates, demyelination, and acute and recall proliferative T cell responses to PLP<sub>139–151</sub>. Th1-type cytokines (IL-2 and IFN-γ), thought to contribute to EAE pathogenesis, were also reduced. Others have previously reported that transmethylation inhibition interferes with gene transcription of several cytokines and attributed this to inefficient arginine methylation of STAT1 (33) and/or of the NF-AT cofactor protein, NIP45 (34). The disease-reducing effects of DZ2002 were unambiguously mediated by suppression of autoreactive T cells, because PLP<sub>139–151</sub>-primed T cells were severely compromised in their ability to adoptively transfer EAE in DZ2002-treated recipients.

In addition to direct effects on activated T cells, inhibition of transmethylation may affect other processes in autoimmune/inflammatory responses. Indeed, we found a moderate impairment in chemokine-induced migration of DZ2002-treated monocytes (data not shown). This finding supports previous studies which showed that transmethylation reactions are required for effective recruitment of human monocytes to chemottractants (35, 36). Thus, the beneficial effects of transmethylation inhibition may encompass reductions in the trafficking and homing of inflammatory cells into the CNS. Also, deimidation (the posttranslational conversion of positively charged arginine to neutral citrulline) and arginine methylation of myelin basic protein are increased in multiple sclerosis (1, 37), and it has been speculated that such posttranslational modifications of autoantigens in this and other autoimmune diseases, such as systemic lupus erythematosus (38), may provoke autoimmune responses. If so, transmethylation inhibition may reduce the availability of the inciting autoantigen(s).

In summary, we demonstrated that transmethylation inhibition suppresses CD4 T cell activation and that a reversible inhibitor of transmethylation effectively blocked EAE induction and, more clinically relevant, ameliorated established EAE. Considering that CD4 T cells are required in almost every autoimmune disease, reversible inhibition of transmethylation may have broad therapeutic potential, and indeed our current preliminary studies with DZ2002 indicate suppression of a lupus-like disease in a spontaneous mouse model.

**Acknowledgments**

We thank Drs. Dennis R. Burton, Bruce Beutler, Laurie Owen, Nicholas R. J. Gascoigne, Kerri Mowen, and Dwight H. Kono for critical reading and review of the manuscript and Michael D. Wilson for statistical assistance.

**Disclosures**

The authors have no financial conflict of interest.

**References**


TRANSMETHYLATION INHIBITION IS BENEFICIAL IN AUTOIMMUNITY


