A monoclonal natural autoantibody that promotes remyelination suppresses central nervous system inflammation and increases virus expression after Theiler’s virus-induced demyelination

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Abstract
We have used an established experimental model of multiple sclerosis to investigate the potential beneficial relationship between natural autoimmunity and remyelination after central nervous system (CNS) demyelination. Intracerebral infection of SJL/J mice with Theiler’s murine encephalomyelitis virus (TMEV) produces chronic, progressive, inflammatory CNS demyelination. Chronically infected SJL/J mice show minimal spontaneous remyelination, which is in part due to a T cell-mediated immune response inhibiting myelin repair. We previously identified a monoclonal natural autoantibody, designated SCH94.03, that promotes remyelination when passively transferred to chronically infected SJL/J mice. The mechanism whereby SCH94.03 promotes remyelination is unknown, although previous reports suggest that natural autoantibodies can modulate immune system function. In this report we demonstrate that treatment with SCH94.03 reduced by 2- to 3-fold the number of CD4⁺ and CD8⁺ T cells infiltrating the CNS of SJL/J mice chronically infected with TMEV, in the absence of global lymphocyte depletion. Associated with the decreased inflammation was a 2- to 3-fold increase in virus antigen expression without a significant increase in viral RNA or virus titers. Treatment with SCH94.03 also suppressed the humoral immune response to a T cell-dependent antigen in chronically infected mice. Immunohistochemical staining showed that SCH94.03 labeled MHC class II-positive dendritic cells in peripheral lymphoid organs. These results are consistent with the proposed immunomodulatory function of natural autoantibodies and suggest that one mechanism whereby SCH94.03 promotes CNS remyelination in chronically infected SJL/J mice is through inhibition of a pathogenic immune response.

Introduction
Immune system dysregulation is an important etiologic factor in autoimmune and immunopathologic diseases (1). Current therapies for diseases associated with immune system dysregulation are designed to suppress a primary immune system component. The deleterious side effects of global immunosuppression dictate that the next generation of therapeutic agents employ a more selective mechanism of action. One approach would be to augment the intrinsic regulatory mechanisms that prevent inappropriate immune system reactivity in normal individuals. Natural or physiological autoantibodies may be one intrinsic component that prevents inappropriate immune system reactivity. Natural autoantibodies are present in the serum of normal individuals, are typically polyreactive with a wide range of self and non-self antigens, share extensive idiotypic cross-reactivity, and are encoded by germline Ig genes (2). Although their function is
unknown, natural autoantibodies can modulate immune system function (3-6). This suggests that manipulation of physiological humoral autoimmunity represents a potential therapy for deleterious immune system reactivity.

We have used an experimental model of central nervous system (CNS) demyelination in SJL/J mice induced by Thiel's murine encephalomyelitis virus (TMEV) to investigate the relationship between humoral autoimmunity and CNS remyelination. Intracerebral infection of SJL/J mice with the Daniel's (DA) strain of TMEV produces chronic, progressive, immune-mediated CNS demyelination that is similar to the chronic progressive form of multiple sclerosis (MS) (7,8). Spontaneous remyelination in chronically infected SJL/J mice is minimal and limited to the periphery of demyelinated lesions (9), in part due to an inhibitory T cell-mediated immune response that can be suppressed by treatment with cyclophosphamide or mAb to CD4 or CD8 (10). In contrast, the humoral immune response is beneficial and can promote CNS remyelination in chronically infected SJL/J mice (11-13). Passive transfer of either antiserum (11) or purified polyclonal IgG (12) from uninfected SJL/J mice injected with normal syngeneic homogenized spinal cord (SCH) into chronically infected SJL/J mice stimulates CNS myelin repair. Using a similar antibody-induction procedure, we identified an IgM mAb, designated SCH94.03, that promotes remyelination when passively transferred into chronically infected SJL/J mice (13). SCH94.03 is a natural autoantibody encoded by germline V H 10-J 1 and V 23-D 16-J 2 Ig genes (14). These studies provide support for the concept that natural or physiological autoimmunity can be beneficial in a CNS demyelinating disease.

On the basis of the postulated immunomodulatory function of natural autoantibodies (2) and the presence of a T cell-mediated immune response in chronically infected SJL/J mice that inhibits remyelination (10), we tested the hypothesis that treatment with SCH94.03 would reduce CNS inflammation in SJL/J mice chronically infected with TMEV. In this report we demonstrate that treatment with SCH94.03 reduced the number of CD4 + and CD8 + T cells infiltrating the spinal cords of chronically infected SJL/J mice, without globally depleting lymphocytes. In addition, chronically infected mice treated with either SCH94.03 or cyclophosphamide had increased TMEV antigen expression and suppressed humoral immune responses. These data support the hypothesis that SCH94.03 has an immunomodulatory effect in SJL/J mice chronically infected with TMEV.

Methods

Mice

Female SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Handling of all animals conformed to the National Institutes of Health and Mayo Clinic institutional guidelines.

Virus

The DA strain of TMEV was used for all virus experiments. The origin and propagation of this virus in tissue culture have been previously described (15). Mice (4-6 weeks old) were inoculated intracerebrally with 2 x 10^5 p.f.u. of TMEV in a 10 μl volume. Virus antigens for ELISA were purified from infected BHK-21 cells by ultracentrifugation on cesium chloride density gradients as described (15).

Antibodies

The production and characterization of mAb SCH94.03 have been previously described (13,14). The hybridoma XXMEN-OE5 secretes an anti-hpopolysaccharide IgM mAb and was purchased from the ATCC (Rockville, MD). We purified SCH94.03 and XXMEN-OE5 IgM from tissue culture supernatant by precipitation in 50% saturated ammonium sulfate. Precipitated IgM was washed once with 50% saturated ammonium sulfate, dissolved in PBS, reprecipitated by dialysis against distilled water at 4°C, solubilized in 2X PBS and stored at -70°C. Purified IgM was checked for purity by SDS-PAGE and quantitated by a μ chain-specific capture ELISA using purified IgM myeloma MOPC104E (Sigma, St Louis, MO) as the reference standard.

Reagents used for flow cytometry (FCM) and immunohistochemistry are listed in Table 1. Phycoerythrin (PE)-labeled streptavidin was from Tago (Burlingame, CA). All remaining reagents were from Jackson Immunoresearch (West Grove, PA).

Treatment protocol

Mice were used at 6-8 months after infection, at which time they showed clinical signs of chronic demyelination characterized by ruffled fur, huddled posture, spasticity, with occasional paralysis and incontinence. Mice were treated with 50 μg of mAb injected i.p. twice weekly for 5 weeks (total dose of 0.5 mg or ~25 mg/kg). Animals in the buffer treatment group were injected with a comparable volume of PBS. For

Table 1. Reagents used for immunostaining

<table>
<thead>
<tr>
<th>Hybridoma/clonemAb</th>
<th>Antigen specificity</th>
<th>Isotype</th>
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<tr>
<td>RM4-5</td>
<td>CD4</td>
<td>rat IgG2a</td>
<td>PharMingen</td>
</tr>
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<td>rat IgG2b</td>
<td>Becton Dickinson</td>
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<td>rat IgG2b</td>
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</tr>
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<td>rat IgG2b</td>
<td>PharMingen</td>
</tr>
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<td>M1/70</td>
<td>CD11b(Mac-1)</td>
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<td>PharMingen</td>
</tr>
<tr>
<td>RA3-6B2</td>
<td>CD45R(B220)</td>
<td>mouse IgG2a</td>
<td>PharMingen</td>
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<td>7-16.17</td>
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<td>ATCC</td>
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<tr>
<td>2.422</td>
<td>FcγR</td>
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<td>Sigma</td>
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cyclophosphamide treatment, mice were injected i.p. with 1 mg weekly for 5 weeks (total dose of 5 mg or ~250 mg/kg).

Spleen and CNS-infiltrating mononuclear cell preparations and FCM analysis

Spleocytes from chronically infected SJL/J mice were prepared for FCM analysis as previously described (16). Mononuclear cells infiltrating the CNS of chronically infected SJL/J mice were isolated as previously described (17) with several modifications. Brains and spinal cords of chronically infected SJL/J mice were aseptically removed and placed in ice-cold Hank's balance salt solution with 10 mM HEPES buffer (HBSS/H). Tissue was dissociated with a Potter-Elvehjem tissue grinder and mononuclear cells were isolated from the CNS tissue homogenate by separation on a Percoll gradient (17). Recovered mononuclear cells were washed three times in HBSS/H, resuspended in FCM buffer (PBS with 0.2% azide and 2.5% FCS), filtered through 30-μm Nytex mesh, and counted by Trypan blue exclusion. We only counted cells with the small round morphology of mononuclear cells. The viability of recovered CNS-infiltrating mononuclear cells was routinely 90–95%.

All FCM staining incubations were done for 1 h at 4°C in the FCM buffer described above unless otherwise indicated. Cells (1–2 × 10^6 per sample) were incubated with 2 μg FITC- or PE-labeled primary antibodies and 1 μg isotype-matched control antibodies. After blocking for 30 min with 2% normal mouse serum, cells were incubated with primary antibodies, washed, and incubated with the appropriate FITC- or PE-labeled secondary antibodies. Appropriately labeled, isotype-matched control primary antibodies were used to determine non-specific staining. Cells were fixed with 4% buffered paraformaldehyde overnight prior to analysis. Samples were analyzed on a FACScan, FACS Vantage or FACStarPlus flow cytometer (Becton Dickinson, Palo Alto, CA), and data were analyzed using the PC-LYSYS software program (Becton Dickinson). A gate was set to analyze cells with forward- and right-angle light scatter patterns characteristic of viable splenic lymphocytes (18), and at least 5000 viable cells were analyzed per sample. The number of surface marker-positive mononuclear cells from individual mice was calculated by multiplying the total number of recovered cells by the percentage of cells positive for a specific marker.

Immunostaining

Freshly isolated spleen, thymus, and inguinal, axillary and cervical lymph nodes from uninfected SJL/J mice and spinal cords from chronically infected SJL/J mice were quickly frozen in isopentane chilled with liquid nitrogen prior to liquid nitrogen storage. Longitudinal spinal cord cryostat sections 10 μm thick were fixed for 20 min in ice-cold 0.1 M phosphate buffer containing 0.5% paraformaldehyde, 0.5% glutaraldehyde, 0.002% calcium chloride, 1.6% glucose and 1% dimethyl sulfoxide. Fixed sections were quenched with ice-cold 0.15 M ethanolamine (pH 7.5) for 20 min, washed in PBS and treated with 1 μg proteinase K in PBS for 30 min at 37°C and 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min at room temperature. After acetylation, slides were dehydrated in ethanol and pre-hybridized with 0.5 mg/ml sonicated salmon sperm DNA, 0.5 mg/ml yeast total RNA and 50 μg/ml yeast tRNA in 50% deionized formamide, 0.6 M NaCl, 20% 10×Denhardt’s solution and 1 mM EDTA for 4 h at room temperature. Slides were hybridized with 35S-labeled 253 nucleotides (3053–3305) or 363 nucleotides (3306–3668) bp cDNA probes corresponding to the VP1 region of the DA strain of TMEV (19). cDNA probes were obtained by double digesting VP1 with KpnI and SalI restriction enzymes and were radiolabeled with nick translation with [α-35S]dATP to specific activities between 0.5 and 0.8 × 10^8 c.p.m./μg DNA. Hybridization was carried out overnight at 37°C in 50% formamide, 20% 10×Denhardt’s solution, 1 mM EDTA, 10 mM dithiothreitol, 0.1% SDS and 20% dextran sulfate containing 0.1 mg/ml sonicated salmon sperm DNA, 0.5 mg/ml total yeast RNA and 50 μg/ml yeast tRNA. After hybridization, slides were washed with 2×SSC containing 1% sodium glycoprophosphatidylcholine and 1×SSC for 5 min. Slides were dried and exposed to X-ray film. Hybridized sections were stained with hematoxylin and eosin.

In situ hybridization for TMEV RNA

Spinal cord cryostat sections from chronically infected SJL/J mice were fixed for 20 min in ice-cold 0.1 M phosphate buffer containing 0.5% paraformaldehyde, 0.5% glutaraldehyde, 0.002% calcium chloride, 1.6% glucose and 1% dimethyl sulfoxide. Fixed sections were incubated with the appropriate FITC- or PE-labeled secondary antibodies. Appropriately labeled, isotype-matched control primary antibodies were used to determine non-specific staining. Cells were fixed with 4% buffered paraformaldehyde overnight prior to analysis. Samples were analyzed on a FACScan, FACS Vantage or FACStarPlus flow cytometer (Becton Dickinson). A gate was set to analyze cells with forward- and right-angle light scatter patterns characteristic of viable splenic lymphocytes (18), and at least 5000 viable cells were analyzed per sample. The number of surface marker-positive mononuclear cells from individual mice was calculated by multiplying the total number of recovered cells by the percentage of cells positive for a specific marker.

Immunostaining

Freshly isolated spleen, thymus, and inguinal, axillary and cervical lymph nodes from uninfected SJL/J mice and spinal cords from chronically infected SJL/J mice were quickly frozen in isopentane chilled with liquid nitrogen prior to liquid nitrogen storage. Longitudinal spinal cord cryostat sections 10 μm thick were fixed with acetone and immunostained with mAb to CD4, CD8α and β, CD45R(B220), Bandeiraea simplicifolia isolecitin B4, or polyclonal rabbit anti-TMEV (15) using the ABC immunoperoxidase procedure previously described (13). Appropriately labeled, isotype-matched control primary antibodies were used to determine non-specific staining. We excited FITC, TR and AMCA fluorochromes with a mercury lamp attached to a Zeiss microscope equipped with filter sets to visualize FITC/TR fluorescence simultaneously (480–497/540–557 nm bandpass exciter filter, 495/560 nm dichroic beam splitter filter, 510–530 nm bandpass/580 nm long pass barrier filter) and AMCA fluorescence separately (G365 nm exciter filter, 395 nm dichroic beam splitter filter, 420 nm long pass barrier filter).

TMEV antigen quantitation

We used a quantitative immunohistochemical analysis to assess TMEV antigen levels in the spinal cords of chronically infected SJL/J mice. Immunostained slides from individual mice were coded, and both the total white matter area and the area with a dark immunoperoxidase reaction product corresponding to the presence of TMEV antigens were measured using an IBAS 2000 Image Analysis System (Kontron, Munich, Germany) attached to an Axiosphot microscope (Carl Zeiss, Thornwood, NY). Data are expressed as the percentage of white matter area with TMEV antigen immunoreactivity. To determine whether this analysis was an accurate representation of the number of cells containing TMEV antigens, we also quantitated the number of TMEV antigen-positive cells in spinal cord cryostat sections from 11 mice treated with SCH94.03, control IgM or PBS. There was a statistically significant correlation (R = 0.79; P < 0.004) between the number of TMEV antigen-positive cells and the area of TMEV antigen immunoreactivity.

In situ hybridization for TMEV RNA

Spinal cord cryostat sections from chronically infected SJL/J mice were fixed for 20 min in ice-cold 0.1 M phosphate buffer containing 0.5% paraformaldehyde, 0.5% glutaraldehyde, 0.002% calcium chloride, 1.6% glucose and 1% dimethyl sulfoxide. Fixed sections were incubated with 1 μg proteinase K in PBS for 30 min at 37°C and 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min at room temperature. After acetylation, slides were dehydrated in ethanol and pre-hybridized with 0.5 mg/ml sonicated salmon sperm DNA, 0.5 mg/ml yeast total RNA and 50 μg/ml yeast tRNA in 50% deionized formamide, 0.6 M NaCl, 20% 10×Denhardt’s solution and 1 mM EDTA for 4 h at room temperature. Slides were hybridized with 35S-labeled 253 nucleotides (3053–3305) or 363 nucleotides (3306–3668) bp cDNA probes corresponding to the VP1 region of the DA strain of TMEV (19). cDNA probes were obtained by double digesting VP1 with KpnI and SalI restriction enzymes and were radiolabeled with nick translation with [α-35S]dATP to specific activities between 0.5 and 0.8 × 10^8 c.p.m./μg DNA. Hybridization was carried out overnight at 37°C in 50% formamide, 20% 10×Denhardt’s solution, 1 mM EDTA, 10 mM dithiothreitol, 0.1% SDS and 20% dextran sulfate containing 0.1 mg/ml sonicated salmon sperm DNA, 0.5 mg/ml total yeast RNA and 50 μg/ml yeast tRNA. After hybridization, slides were washed with 2×SSC containing 1% sodium glycoprophosphatidylcholine and 1×SSC for 5 min. Slides were dried and exposed to X-ray film. Hybridized sections were stained with hematoxylin and eosin.
thiosulfate, 0.05% sodium pyrophosphate and 0.1% 2-mercaptoethanol for 2 h at 55°C and 1 h at room temperature. Slides were further washed in 1×SSC containing 1% sodium thiosulfate, 0.05% sodium pyrophosphate and 0.1% 2-mercaptoethanol for 1 h at 55°C, 30 min at room temperature, 1 h at 37°C, rinsed sequentially in 2×SSC containing 50% formamide for 15 min and 1×SSC for 10 min, and dehydrated in a graded series of ethanol containing 0.3 M ammonium acetate. Slides were air dried, immersed in NTB-2 emulsion (Eastman Kodak, New Haven, CT), and exposed for 1–3 days at 4°C before developing and fixing. Slides were counterstained with hematoxylin & eosin.

We quantitated TMEV RNA in spinal cord sections using the IBAS system described above. We measured both the total white matter area and the number of silver grains on spinal cord sections from individual mice. Data are expressed as the number of grains per area of white matter.

**Virus plaque assay**

Infectious TMEV titers were determined in clarified CNS homogenates by plaque assay as previously described (20). Brains and spinal cords were removed aseptically from mice, placed in 10 volumes of DMEM and stored at −70°C until use. The tissue was homogenized, sonicated three times for 20 s and clarified by centrifugation. Plaque assays were performed in duplicate on coded samples. The lower detection limit was 250 p.f.u./g CNS tissue.

**Humoral immune responses**

To elicit secondary IgG immune responses against T cell-dependent antigens, chronically infected SJL/J mice were injected i.p. with 100 μg chicken γ-globulin (CGG) and 100 μg ovalbumin (OVA) in PBS 10 days after the initiation of treatment with SCH94.03, control IgM, PBS or cyclophosphamide. Mice were boosted with an identical dose 14 days later. To elicit a primary IgM immune response to a T cell-independent antigen, chronically infected mice were injected i.p. with 100 μg trinitrophenyl (TNP)-Ficoll 28 days after the initiation of treatment. Mice were bled by intracardiac puncture 39–40 days after the initiation of treatment. Blood was allowed to clot overnight at 4°C and serum was aliquoted and stored at −70°C.

Serum antigen-specific Ig levels were determined by an ELISA for CGG, OVA and TNP-Ficoll antibody responses. 96-well polyvinylchloride microtiter plates were coated with 1 μg antigen/well in 0.1 M carbonate buffer, pH 9.5. We used TNP-BSA as the coating antigen to assess TNP-Ficoll antibody responses. Parallel control plates coated with 1 μg BSA/well were used to determine antibody reactivity with BSA. Plates were blocked with 5% non-fat dry milk and serum from individual mice was diluted in 5-fold increments from 1:100 to 1:312,500 in 0.2% BSA and incubated for 4 h on antigen-coated plates. We detected bound IgG and IgM with biotinylated goat anti-mouse IgG and biotinylated goat anti-mouse IgM respectively, and streptavidin conjugated to alkaline phosphatase. We used p-nitrophenylphosphate as the enzyme substrate and 405 nm as the absorbance wavelength. We used the antibody titer for statistical comparisons between treatment groups. This unit is defined as the serum dilution producing a half-maximal A<sub>405</sub> in an antigen-specific ELISA and calculated with regression analysis through the linear portion of the titration curves.

For TMEV-specific IgG responses, polystyrene microtiter plates were coated with 0.5 μg purified TMEV antigen/well. Plates were blocked with 1% BSA and bound IgG was detected as described above. The TMEV-specific IgG response data are presented as the A<sub>405</sub> of a 1:5000 serum dilution. This serum dilution fell on the linear portion of the titration curves generated using pooled serum from mice treated with either SCH94.03 or PBS.

**Statistical analyses**

We used an unpaired Student's t-test assuming unequal variances to evaluate differences between treatment groups, unless otherwise indicated. Correlation significance was determined by a one-way analysis of variance. Statistical results were considered significant when P < 0.05.

**Results**

**CNS-infiltrating mononuclear cells in chronically infected SJL/J mice treated with SCH94.03**

To determine whether treatment with SCH94.03 reduced CNS inflammation, we assessed the number of CNS-infiltrating 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Total no. of surface marker positive CNS-infiltrating mononuclear cells (×10&lt;sup&gt;5&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>CD5&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>PBS</td>
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<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>Control IgM</td>
<td>12</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>SCH94.03</td>
<td>12</td>
<td>2.3 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

SJL/J mice chronically infected with TMEV were injected i.p. with a total dose of 0.5 mg SCH94.03, control IgM or an equivalent volume of PBS, divided into twice weekly doses for 5 weeks. For control IgM, MOPC104E and XXMEN-OE5 were used. The data are a composite of two independent experiments and are presented as the mean ± SEM, where N indicates the number of mice.

<sup>a</sup>Cell numbers were calculated by multiplying the percentage of positive cells assessed by FCM with the total number of mononuclear cells isolated from brain and spinal cord homogenates of individual mice by Percoll gradient separation.

<sup>b</sup>P < 0.00001 when compared with combined control IgM and PBS data.

<sup>P</sup> < 0.00005 when compared with combined control IgM and PBS data.

<sup>OP</sup> < 0.007 when compared with combined control IgM and PBS data.

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**Table 2. FCM analysis of mononuclear cells infiltrating the CNS of chronically infected SJL/J mice**
mononuclear cells in chronically infected SJL/J mice treated with SCH94.03. FCM analysis of mononuclear cells directly isolated from the brains and spinal cords of chronically infected SJL/J mice indicated that SCH94.03 treatment reduced the total number of CD5⁺, CD4⁺ and CD8⁺ T cells infiltrating the CNS (Table 2). Mice treated with SCH94.03 had a 2- to 3-fold reduction in CD5⁺ T cells, a 2-fold reduction in CD4⁺ T cells and a 2- to 3-fold reduction in CD8⁺ T cells compared with mice treated with control IgM or PBS. Treatment with SCH94.03 also reduced the number of CNS-infiltrating CD45R(B220)⁺ B cells, although these cells made up <5% of the total mononuclear cell population isolated by Percoll gradient separation, consistent with a previous report (21). In the one experiment where the total number of CNS-infiltrating CD11b(Mac-1)⁺ macrophages was determined, there were fewer macrophages in mice treated with SCH94.03 (3.1 ± 0.5x10⁵; mean ± SEM; n = 6) compared with mice treated with control IgM or PBS (4.7 ± 0.7x10⁵; n = 11), although this reduction was not statistically significant (P = 0.07). We concluded from these data that treatment with SCH94.03 decreased CNS inflammation in SJL/J mice chronically infected with TMEV.

We also examined by immunohistochemistry the localization of CNS inflammatory cells in chronically infected SJL/J mice treated with SCH94.03 (Fig. 1). Although SCH94.03 reduced CNS inflammation, the distribution of the remaining inflam-

![Image](https://academic.oup.com/intimm/article-abstract/8/1/131/859448/10.1093/intimm/dix044)
ary mononuclear cells did not differ from mice treated with control IgM or PBS. Consistent with previous observations (17), CD4+ T cells were located primarily in penvascular areas, although some CD4+ cells were present in the parenchyma (Fig. 1A). In contrast, CD8+ T cells were found primarily in the parenchyma (Fig. 1B). The few CD45R(B220)+ B cells present in the CNS of chronically infected mice were restricted to the penvascular areas (Fig. 1C), whereas macrophages identified by reactivity with *Bandeiraea simplicifolia* isolecitin B4 were present throughout white matter lesions (Fig. 1D).

Peripheral lymphocyte populations in chronically infected SJL/J mice treated with SCH94.03

We examined whether the decreased number of CNS-infiltrating mononuclear cells in chronically infected SJL/J mice treated with SCH94.03 was secondary to global lymphocyte depletion. The total number of splenic B and T cells was similar in mice treated with SCH94.03 compared with mice treated with control IgM or PBS. Chronically infected SJL/J mice treated with SCH94.03 had 8.3 ± 1.8x10^7 (n = 6) splenic CD45R(B220)+ B cells compared with 5.9 ± 0.6x10^7 (n = 11) in mice treated with control IgM or PBS (P > 0.2). Similarly, mice treated with SCH94.03 had 9.3 ± 1.3x10^7 splenic CD5+ T cells, 6.9 ± 1.0x10^7 splenic CD4+ T cells and 2.3 ± 0.3x10^7 splenic CD8+ T cells, compared with 7.5 ± 0.9x10^7 splenic CD5+ T cells, 5.6 ± 0.6x10^7 splenic CD4+ T cells and 1.8 ± 0.2x10^7 splenic CD8+ T cells in mice treated with control IgM or PBS (P > 0.2 for all). These data indicated that SCH94.03 treatment promoted CNS remyelination without global lymphocyte depletion, in contrast to *in vivo* treatment with mAb to CD4 or CD8 (10).

Virus titers in the CNS of chronically infected mice treated with SCH94.03

To assess TMEV levels after treatment with SCH94.03, we measured infectious virus titers in the CNS of chronically infected SJL/J mice. Using a standard plaque assay, infectious TMEV was detected in two of six mice treated with SCH94.03, three of six mice treated with control IgM and three of four mice treated with PBS (P > 0.4 using the χ²-test). In animals with detectable infectious virus, the two mice treated with SCH94.03 had an average of 600 p.f.u./g CNS tissue compared with 2200 ± 900 p.f.u./g CNS tissue in the six mice treated with control IgM or PBS (P > 0.1 using Student's t-test). Therefore, treatment with SCH94.03 did not significantly change the level of infectious virus in the CNS of chronically infected SJL/J mice.

TMEV antigen expression in the CNS of chronically infected mice treated with cyclophosphamide or SCH94.03

Although the presence of infectious virus can predict susceptibility to TMEV-induced demyelination in some cases (22), local virus antigen and RNA production correlates better with the presence or absence of demyelination (23–36). Therefore, we examined whether the increased CNS remyelination and decreased CNS inflammation after treatment with SCH94.03 were associated with a change in TMEV antigen expression in the spinal cords of chronically infected mice. Cells containing virus antigen as identified by immunohistochemistry were present primarily in white matter either within inflammatory lesions or in directly adjacent tissue (Fig. 2A). When we quantitated TMEV antigen expression in spinal cord sections, mice treated with SCH94.03 had a 2- to 3-fold increase in the
expression of virus antigen compared to mice treated with control IgM or PBS (Table 3). Although the increase in TMEV antigen expression in chronically infected SJL/J mice treated with SCH94.03 without an increase in virus titers may initially seem contradictory, discrepancies between virus titers and virus antigen have previously been observed after infection with TMEV (24,25). We concluded from these data that the decrease in CNS inflammation after treatment with SCH94.03 was associated with an increase in TMEV antigen expression in chronically infected SJL/J mice.

Rodriguez and Lindsley have previously demonstrated a qualitative increase in the number of TMEV antigen-positive cells in the spinal cords of chronically infected SJL/J mice after treatment with cyclophosphamide, a global immunosuppressive agent that promotes CNS remyelination (10). To provide a quantitative analysis of this observation, we assessed TMEV antigen expression in chronically infected SJL/J mice treated with cyclophosphamide by quantitative immunohistochemistry. Chronically infected SJL/J mice treated with cyclophosphamide had a 2- to 3-fold increase in TMEV antigen expression compared with mice treated with PBS, similar to the increase seen after treatment with SCH94.03 (Table 3). These data confirm the observation that immunosuppression can be associated with an increase in both CNS remyelination and TMEV antigen expression.

**TMEV RNA expression in the CNS of chronically infected mice treated with cyclophosphamide or SCH94.03**

We also examined TMEV RNA expression in spinal cord cryostat sections from chronically infected SJL/J mice by quantitative in situ hybridization. Consistent with the immunohistochemical staining for TMEV antigens, cells containing TMEV RNA were found primarily in white matter lesions or in directly adjacent tissue (Fig. 2B). When we quantitated TMEV RNA expression, mice treated with SCH94.03 or cyclophosphamide had more virus RNA in their spinal cords than control animals, although these differences were not statistically significant (Table 3). However, when we compared TMEV antigen and RNA expression in individual mice, there was a moderate but statistically significant correlation ($R = 0.49, P < 0.01$) between the level of virus antigen and RNA expression, consistent with previous observations (27,28).

**Humoral immune responses in chronically infected mice treated with cyclophosphamide or SCH94.03**

We assessed whether the increased TMEV antigen expression in the CNS of mice treated with SCH94.03 was associated with a change in the serum anti-TMEV IgG levels. At a 1:5000 serum dilution in a TMEV-specific ELISA, serum from mice treated with SCH94.03 had an $A_{405}$ of 0.821 ± 0.034 (n = 20) compared with 0.841 ± 0.020 (n = 37) for mice treated with control IgM or PBS. 0.881 ± 0.026 (n = 8) for mice treated with cyclophosphamide and 0.11 ± 0.001 (n = 3) for uninfected SJL/J mice. These results support the observations that the virus-specific humoral immune response plays a limited role in the pathogenesis of TMEV-induced demyelination (29–32).

The absence of antibody suppression with cyclophosphamide treatment suggested that the TMEV-specific IgG measured represented predominantly pre-existing antibody prior to the initiation of therapy. Therefore, to verify the immunosuppressive capability of the cyclophosphamide dose used and to assess the potential immunomodulatory effect of SCH94.03 on humoral immune responses, we injected chronically infected mice with both T cell-dependent and T cell-independent antibodies after the initiation of therapy. We measured secondary IgG responses to the T cell-dependent protein antigens CGG and OVA and primary IgM responses to the T cell-independent antigen TNP-Ficoll in chronically infected SJL/J mice (Fig 3). Treatment with cyclophosphamide completely suppressed both the TNP-specific IgM and OVA-specific IgG responses to Ig levels seen in naive mice, whereas the CGG-specific IgG response was only partially suppressed. In contrast, although treatment with SCH94.03 significantly inhibited the IgG response to CGG, the antibody responses to OVA and TNP-Ficoll after treatment with SCH94.03 were not statistically different than chronically infected SJL/J mice treated with control IgM or PBS. These results demonstrated that SCH94.03 could have an in vivo immunomodulatory effect on antibody production in the absence of global immunosuppression.

**SCH94.03 immunoreactivity in peripheral lymphoid organs**

The observation that SCH94.03 had immunomodulatory effects on T cell populations in chronically infected SJL/J mice suggested that SCH94.03 might inhibit T cell activation or function directly. To assess the potential interactions between SCH94.03 and cells involved in lymphocyte activation, we examined SCH94.03 immunoreactivity in peripheral lymphoid organs using multi-color immunofluorescence staining (Fig. 4). SCH94.03 did not react with T cells in spleen, thymus or lymph nodes cryostat sections, consistent with previous FCM results (33). SCH94.03 reactivity toward B cells was not

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**Table 3. Quantitative analysis of TMEV antigen and RNA expression in the CNS of chronically infected SJL/J mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>TMEV antigen expression$^a$</th>
<th>TMEV RNA expression$^b$</th>
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<tbody>
<tr>
<td>PBS</td>
<td>7</td>
<td>0.08 ± 0.03</td>
<td>1073 ± 282</td>
</tr>
<tr>
<td>Control IgM</td>
<td>8</td>
<td>0.11 ± 0.02</td>
<td>1187 ± 349</td>
</tr>
<tr>
<td>SCH94 03</td>
<td>8</td>
<td>0.24 ± 0.04$^c$</td>
<td>1702 ± 839$^e$</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>8</td>
<td>0.22 ± 0.05$^d$</td>
<td>2122 ± 534$^e$</td>
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</table>

SJL/J mice chronically infected with TMEV were treated as described in the Table 2 legend. Mice treated with cyclophosphamide were injected i.p. with 1 mg weekly for 5 weeks. The data are a composite of two independent experiments and are presented as the mean ± SEM, where N indicates the number of mice. The total area of white matter examined was not statistically different between treatment groups.

$^a$Percentage of white matter area with TMEV antigen immunoreactivity was determined by a quantitative immunohistochemical analysis of spinal cord cryostat sections immunostained with polyclonal rabbit anti-TMEV sera.

$^b$Number of grains/mm$^2$ white matter was determined by a quantitative in situ analysis of spinal cord cryostat sections hybridized with an $^{35}$S-labeled VP-1 probe.

$^c$P < 0.006 when compared with combined control IgM and PBS data.

$^d$P < 0.04 when compared with combined control IgM and PBS data.

$^e$P > 0.1 when compared with combined control IgM and PBS data.
sheath, although they were not as numerous as in lymph nodes. Dendritic cells in peripheral lymphoid organs express MHC class II antigens (34), whereas mouse T cells do not express class II antigens. Three color immunofluorescent staining of lymph node sections showed that paracortical lymph node cells which labeled with SCH94.03 did not express the pan-T cell marker CD5 (Fig. 4C), but did express MHC class II (Fig. 4D). These data indicated that SCH94.03 labeled MHC class II-positive dendritic cells in peripheral lymphoid organs.

Discussion

We have postulated two potential mechanisms for SCH94.03-mediated CNS remyelination after TMEV-induced demyelination (35). The first is a direct effect of SCH94.03 on oligodendrocytes, glial precursors, or cells that secrete factors necessary for oligodendrocyte survival or differentiation. The second mechanism is based on an indirect effect, where SCH94.03 suppresses an inhibitory immune response. Rodriguez and Lindsey demonstrated that an immune response involving both CD4+ and CD8+ T cells prevents spontaneous remyelination in SJL/J mice chronically infected with TMEV (10). Here we show that treatment with SCH94.03 reduced the number of CD4+ and CD8+ T cells infiltrating the CNS of chronically infected mice in the absence of global lymphocyte depletion. Although treatment with SCH94.03 did not completely suppress CNS inflammation, the 2- to 3-fold decrease in the number of CNS inflammatory cells was similar in magnitude to the extent of CNS remyelination promoted by SCH94.03 (13). This suggests that one mechanism whereby SCH94.03 promotes CNS remyelination is through suppression of a pathogenic immune response.

Definitive proof that SCH94.03 modulates an inhibitory immune response awaits identification and characterization of the antigenic targets and effector mechanisms of the immune response preventing spontaneous remyelination in chronically infected SJL/J mice. The decreased inflammation and increased virus expression in the CNS of chronically infected SJL/J mice after treatment with SCH94.03 suggests that one component inhibiting remyelination might be a T cell-mediated immune response directed against virus antigens, similar to the pathogenesis of TMEV-induced demyelination (7,8,36). Although both virus-specific delayed-type hypersensitivity (37) and cytotoxic T cell responses (38) have been implicated in TMEV-induced CNS demyelination, apart from the report of Lindsey and Rodriguez (10), no studies have directly examined the effector mechanisms and antigenic targets of the immune response preventing spontaneous remyelination in SJL/J mice after chronic infection with TMEV. The observation that mice deficient in Bcr2 microglobulin demonstrate extensive spontaneous remyelination after chronic infection with TMEV (39) suggests that CD8+ T cells play a critical inhibitory role. We are currently using mouse models with selective genetic immunodeficiencies to further define the immunologic processes preventing remyelination after chronic TMEV infection.

The presence of TMEV antigen and RNA in the CNS of chronically infected mice treated with SCH94.03 or cyclophos-
Fig. 4. Immunostaining of lymphoid interdigitating dendritic cells with SCH94.03 (A) Immunoperoxidase staining of cells with a dendritic morphology in the paracortical region of lymph nodes using SCH94.03. The brown reaction product indicates immunoreactivity (×375) (B) Same field and staining method as in (A) using a control IgM (×375) (C) Immunofluorescent staining of mouse lymph node paracortex with rat anti-CD5 (red) and SCH94.03 (green). The photograph was taken using fluorescence filters for simultaneous red/green visualization (×700) (D) Same field as (C) stained with biotinylated mouse anti-MHC class II monoclonal antibody and AMCA-streptavidin. The cell in the center of (C) and (D) is stained with both SCH94.03 and anti-class II, but not with anti-CD5 (×700) Sections (A) and (B) were counterstained with Mayer’s hematoxylin.

Pharmacology indicates that remyelination occurs despite the presence of a persistent virus infection.Patrick et al. also demonstrated persistent TMEV antigen expression and infectious virus in the CNS of chronically infected SJL/J mice treated with SCH IgG (25), a polyclonal Ig preparation that promotes remyelination (12). Treatment with SCH IgG had a minimal effect on the number of virus-antigen-positive cells in the CNS of chronically infected mice (25). In contrast, we observed an increase in TMEV antigen expression after treatment with SCH94.03. Treatment with SCH IgG also increased the serum levels of TMEV-specific IgG (25), whereas mice treated with SCH94.03 had levels of TMEV-specific IgG similar to controls. Although SCH IgG and SCH94.03 were generated using a similar antibody induction protocol, the inherent differences between a polyclonal IgG reagent and a monoclonal IgM antibody make it difficult to directly compare the effects of SCH IgG and SCH94.03. Nevertheless, the observation of increased virus antigen expression in the spinal cords of mice treated with SCH94.03 or cyclophosphamide support the conclusion that clearance of viral antigen or infectious virus is not a prerequisite for myelin repair and indicate that an increase in viral antigen expression does not
inhibit remyelination. This may have relevance to human demyelinating diseases and implies that if MS is triggered by an infectious agent, elimination of the pathogen is not required for CNS repair.

The demonstration that treatment with SCH94.03 decreased CNS inflammation, increased TMEV expression, and suppressed humoral immune responses during chronic viral infection supports the postulated immunomodulatory function of natural autoantibodies (2). Natural autoantibodies can suppress the anti-acetylcholine receptor antibody response in an autoimmune model of myasthenia gravis (3), prevent the development of diabetes in non-obese diabetic mice (4) and alter the B cell repertoire in normal mice (5,6). Although the mechanisms behind these immunomodulatory effects are unknown, anti-idiotypic interactions have been postulated based on the observation that natural autoantibodies share an extensive network of cross-reactive idiotypes (40). These idiotypic interactions are not limited to Ig idiotypes, as natural autoantibodies have been shown to react with defined TCR idiotypic interaction (42). On the basis of these observations, SCH94.03 might decrease CNS inflammation by inhibiting T cell activation and thereby alter recruitment of T cells into the CNS or indirectly promote lymphocyte migration from the CNS. Although SCH94.03 did not react with T cells directly, this hypothesis is consistent with the dendritic cell reactivity of SCH94.03 in peripheral lymphoid organs. We are currently testing the hypothesis that SCH94.03 disrupts the complex signaling interaction between T cells and dendritic cells using both in vivo and in vitro model systems. Further studies designed to identify the mechanisms of natural autoantibody-mediated immunomodulation may suggest potential therapies to selectively manipulate immune system function in diseases that are associated with deleterious immunoreactivity, such as MS.

Acknowledgements

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AMCA</td>
<td>aminomethylcoumarin</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>Daniel's strain of TMEV</td>
</tr>
<tr>
<td>FCN</td>
<td>flow cytometry</td>
</tr>
<tr>
<td>CGG</td>
<td>chicken y-globulin</td>
</tr>
<tr>
<td>HBSS/H</td>
<td>Hank's balanced salt solution with HEPES buffer</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>SCH</td>
<td>homogenized spinal cord</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler's murine encephalomyelitis virus</td>
</tr>
<tr>
<td>TNP</td>
<td>trinitrophenyl</td>
</tr>
<tr>
<td>TR</td>
<td>Texas red</td>
</tr>
</tbody>
</table>

References

23. Chamorro, M., Aubert, C. and Braicu, M. 1986. Demyelinating...
lesions due to Theiler’s virus are associated with ongoing central nervous system infection. *J. Virol.* 57:992


