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Generation of a Protective T-Cell Response Following Coronavirus Infection of the Central Nervous System Is Not Dependent on IL-12/23 Signaling

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ABSTRACT

The functional role of IL-12 and IL-23 in host defense and disease following viral infection of the CNS was determined. Instillation of mouse hepatitis virus (MHV, a positive-strand RNA virus) into the CNS of mice results in acute encephalitis followed by a chronic immune-mediated demyelinating disease. Antibody-mediated blocking of either IL-23 (anti-IL-23p19) or IL-12 and IL-23 (anti-IL-12/23p40) signaling did not mute T-cell trafficking into the CNS or antiviral effector responses and mice were able to control viral replication within the brain. Therapeutic administration of either anti-IL-23p19 or anti-IL-12/23p40 to mice with viral-induced demyelination did not attenuate T-cell or macrophage infiltration into the CNS nor improve clinical disease or diminish white matter damage. In contrast, treatment of mice with anti-IL-12/23p40 or anti-IL-23p19 resulted in inhibition of the autoimmune model of demyelination, experimental autoimmune encephalomyelitis (EAE). These data indicate that (1) IL-12 and IL-23 signaling are dispensable in generating a protective T-cell response following CNS infection with MHV, and (2) IL-12 and IL-23 do not contribute to demyelination in a model independent of autoimmune T-cell–mediated pathology. Therefore, therapeutic targeting of IL-12 and/or IL-23 for the treatment of autoimmune diseases may offer unique advantages by reducing disease severity without muting protective responses following viral infection.

INTRODUCTION

Interleukin (IL-23) and IL-12 are heterodimeric proteins that exhibit many similar structural as well as functional properties (45). Both IL-23 (p19/p40) and IL-12 (p35/p40) share an identical p40 subunit, and receptors for IL-23 and IL-12 utilize the common IL-12Rβ1 chain (46). Moreover, signaling by these cytokines often elicits similar and overlapping immune responses (45,46). For example, both IL-12 and IL-23 are considered important in amplifying T-cell responses including proliferation and cytokine secretion following specific antigenic challenge (8,27,31,33,41,53,65). Expression of IL-12 is associated with the development of

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Th-1 responses characterized by IFN-γ secretion from antigen-specific T lymphocytes in response to infection with intracellular pathogens such as viruses, suggesting an important role in host defense. Experimental infection of mice deficient in either IL-12 or IFN-γ signaling with pathogens including herpes simplex virus, murine cytomegalovirus, respiratory syncytial virus, and measles virus results in increased susceptibility to disease, highlighting the importance of the IL-12 and/or IFN-γ signaling axis in antiviral defense in these animal models of disease (3,13,22). However, humans deficient in their ability to either produce or respond to IFN-γ as a result of mutations in IFN-γ signaling receptor, IL-12p40 subunit, or IL-12 receptor reveal increased vulnerability to select intracellular pathogens including mycobacterium and *Salmonella*, yet display susceptibility to only a limited number of RNA or DNA viruses (9,10,37,44). These findings indicate a more important role for IL-12 and IFN-γ in defense following viral infection of mice, while these factors may be dispensable in generating a protective antiviral response in humans. Expression of IL-23 correlates with the development and expansion of Th-17 cells, and is often associated with mouse models of autoimmune inflammatory diseases including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (5,8,42,50). Recent studies have revealed potentially important roles for IL-23 in host defense in response to infectious agents, suggesting protection can occur in an IL-12–independent manner that may be related to IL-23 signaling (18,20,35,63). However, the functional significance of IL-23 signaling in response to viral infection is not well defined and highlights paucity in our understanding of how IL-23 may tailor specific immune responses resulting in virus-specific T lymphocytes essential in effective host defense.

Intracranial infection of mice with mouse hepatitis virus (MHV) results in an acute encephalomyelitis followed by a chronic demyelinating disease (1,39). The acute stage of disease is characterized by viral infection of glial cells and widespread growth throughout the parenchyma. Virus-specific $CD4^+$ and $CD8^+$ T cells are generated in draining cervical lymph nodes (dCLN) and traffic into the CNS where the viral burden is reduced (40). However, virus persists within the CNS, primarily in white matter tracts, and animals will often develop an immune-mediated demyelinating disease with pathology similar to the human demyelinating disease multiple sclerosis (MS). Both T cells and macrophages are important in amplifying the severity of myelin damage in mice persistently infected with MHV (60–62). Indeed, blocking expression of the CXC chemokine ligand 10, CXCL10, or CC chemokine ligand 5, CCL5, in mice persistently infected with MHV and undergoing chronic demyelination results in a marked improvement in clinical disease that is associated with reduced accumulation of T cells and macrophages within the CNS and limited spread of demyelination (15,36). Unlike Theiler's murine encephalomyelitis virus, in which autoreactive T cells specific for myelin antigens are elicited via epitope spreading and participate in the pathogenesis of disease, the generation of T cells reactive to myelin epitope(s) during chronic disease in MHV-infected mice is limited and not thought to substantially contribute to demyelination (7,24). While adoptive transfer of T cells from MHV-infected rats to naãve recipients results in inflammatory lesions (59), no evidence of a similar response in mice has been reported.

A protective immune response to MHV infection during acute disease is characteristic of a Th-1 response and is associated with robust IFN-γ secretion and cytolytic activity by virus-specific T cells (2,48,55). However, the signaling mechanisms responsible for eliciting IFN-γ–secreting T cells remain elusive. For example, infection of mice deficient in either the IL-12p40 or p35 chain (IL-12–deficient mice) with hepatotropic strains of MHV resulted in a robust Th-1 response characterized by high IFN-γ levels and muted secretion of IL-4 (51). These findings suggest that alternative pathways exist in which IFN-γ production by virus-specific T cells occurs independently of IL-12 expression. Therefore, the present study was undertaken to further evaluate how IL-12 and/or IL-23 signaling contributes to host defense in response to MHV infection of the CNS. Through use of antibodies specific for either IL-23 (anti-IL-23p19) or reactive to both IL-12/23 (anti-IL-12/23p40), data are presented that support and extend previous findings demonstrating an important role for IL-23 in inflammatory autoimmune demyelinating disease, as treatment with either anti-IL-23p19 or anti-IL-12/23p40 reduced clinical disease severity in mice immunized against myelin basic protein (MBP). However, blocking IL-23 and/or IL-12 signaling did not affect the generation or trafficking of MHV-specific $CD4^+$ or $CD8^+$ T cells into the CNS following infection, nor alter viral clearance or reduce the severity of white matter damage when compared to control mice. Therefore, these data demonstrate that protective immune responses to MHV infection occur independently of either IL-12 or IL-23, suggesting that alternative mechanisms exist by which virus-specific T cells are generated. Furthermore, these findings highlight that muting IL-23 signaling does not dampen the ability to generate a protective cellular immune response following infection of mice with MHV.

MATERIALS AND METHODS

Antibodies

A neutralizing rat monoclonal antibody to mouse IL-23p19 (CNTO 209) was generated by immunizations

with a mixture of DNA and protein immunogens. For mouse IL-23 protein production, individual mammalian expression plasmids for mouse p40 and p19 subunits were mixed at a 2:1 mass ratio for p19:p40, and used to transiently transfect HEK 293E cells. To aid with purification, a His tag was placed at the C-terminus of the p19 protein and following transfection, conditioned medium was harvested and standard immobilized metal affinity chromatography utilized to isolate the His containing heterodimeric IL-23 away from contaminating proteins including free p40. The purified fraction was then concentrated and dialyzed into PBS. RT-PCR was used to amplify mouse IL-23 p19 cDNA, which was then inserted into a mammalian expression vector containing the human cytomegalovirus immediate early promoter. Sprague-Dawley rats were immunized with two successive intradermal administrations of plasmid IL-23 p19 DNA in both ears on days 0 and 14 (15 μg/ear). The rats then received three subsequent subcutaneous injections of recombinant mouse IL-23 protein (50 μg on day 28, 40 μg on day 85, and 15 μg on day 343), followed by a subcutaneous injection with Titermax adjuvant (Sigma, St. Louis, MO) (15 μg on day 375), and a simultaneous intradermal administration without Titermax (15 μg on day 375). A final booster injection of 20 μg of protein was administered IV 4 d prior to harvest (day 377). Fusion of rat splenocytes to mouse myeloma cells was performed by conventional hybridoma techniques and standard EIA methods were used to identify clones secreting IL-23–specific antibodies. Positive clones were subcloned twice by limiting dilution. Negative control rat IgG was purchased (Jackson Immuno Research, West Grove, PA). Negative control mouse IgG (CNTO 1322) was generated by Centocor. Neutralizing rat anti-mouse IL-12/23p40 (C17.8) and rat anti-mouse IL-12p35 (C18.2) were generous gifts of Dr. Giorgio Trinchieri and the Wistar Institute (Philadelphia, PA). Ascites was generated at Harlan Bioproducts (Indianapolis, IN) and antibodies were purified by protein G affinity chromatography. CNTO 3913 is a chimeric antibody constructed by Centocor using variable regions from a neutralizing rat anti-mouse IL-12/23p40 mAb generated at Centocor through immunization of Sprague-Dawley rats with recombinant mouse IL-12 (R&D Systems, Minneapolis, MN, USA) in adjuvant, followed by standard hybridoma cell fusion methods. Functional variable region genes were identified from the hybridoma cell line, and the heavy chain and light chain variable region genes were cloned into murine IgG_{2a} and kappa expression vectors, respectively, to generate a chimeric rat/mouse anti-IL12/23p40 mAb (CNTO 3913).

IL-12 and IL-23 ELISA

Mouse IL-12, IL-23, and p40 (0.5 μg/mL) were coated overnight on Nunc Maxisorp plates in PBS. After the plates were washed and blocked, anti-IL-23p19 antibody (CNTO 209, Centocor) was titrated and allowed to bind for 2 h. Bound protein was detected using 1:5000 HRP-conjugated donkey anti-rat IgG antibody (Jackson Immuno Research) followed by substrate.

IL-12 and IL-23 neutralization

Single-cell suspensions were prepared from spleens of C57BL/6 mice, and 2.5×10^6 cells/mL were cultured in complete RPMI with 10 U/mL rhIL-2 (PeproTech, Rocky Hill, NJ, USA) and 1 ng/mL mouse IL-23 or IL-12 (R&D Systems), either alone or pre-incubated with anti-IL-23p19 (CNTO 209), anti-IL-12p35 (C18.2), or anti-IL-12/23p40 (C17.8 or CNTO 3913) antibodies. Rat IgG (Jackson ImmunoLabs) and mouse IgG (CNTO 1322, Centocor) were also used as negative controls. Cell cultures were incubated for 3 d. Supernatants were collected and analyzed for IL-17 or IFN-γ protein by ELISA (R&D Systems) per the manufacturer's instructions.

EAE analysis and mice

Female B10.PL mice (Jackson Laboratories, Bar Harbor, ME) from 6–8 wk of age were used. Mice were injected SC over four sites on the back with a total of 100 μL of complete Freund's adjuvant (CFA) combined with 200 μg guinea pig-myelin basic protein (Sigma). Mice also received 200 ng pertussis toxin (List Biological, Campbell, CA) IP in 0.2 mL PBS at the time of immunization and 48 h later. Mice received three once weekly IP injections of either PBS or 20 mg/kg anti-IL-12/23p40 (C17.8), anti-IL-23p19 (CNTO 209), or rat IgG (Jackson ImmunoLabs) antibodies starting the day prior to EAE induction. Animals that demonstrated clinical signs were scored as follows: limp tail or waddling gait with tail tonicity = 1; waddling gait with limp tail (ataxia) = 2; ataxia with partial limb paralysis = 2.5; full paralysis of one limb = 3; full paralysis of one limb with partial paralysis of second limb = 3.5; full paralysis of two limbs = 4; moribund = 4.5; and death = 5. EAE incidence, median time to onset, disease burden over time, highest clinical signs during acute EAE, number of EAE relapses, and severity of EAE relapses $\pm$ SEM are described. Relapses were defined by a full point drop in clinical sign score sustained for at least 2 observed days, followed by a full point increase in clinical sign score sustained for at least 2 observed days. Scores for animals that were sacrificed or scored a 5 were not included in the mean daily analysis of clinical signs for the remainder of the experiment. Incidence of EAE and mortality were compared between groups using Fisher's exact test. Time to EAE onset was evaluated using life table methods and the log-rank test was used for comparisons between groups. The number of relapses and highest clinical scores during acute and relapse phases were analyzed us-

ing the Cochran-Mantel-Haenszel ANOVA statistic. These analyses were conducted using the SAS System for Windows, V8 (SAS Software, Cary, NC). The weighted mean clinical score was calculated for individual animals to determine the disease burden over time. This area-under-the-curve type analysis determines the average daily disease burden for each animal over the entire study. The units of this end-point are the same as the units of an individual daily clinical score. Disease burden over time was analyzed via a standard linear model and analysis of variance (ANOVA). Pairwise comparisons among the groups were evaluated. Disease burden over time analyses were performed using the R software environment (a). For all analyses, p values <0.05 were considered significant. No adjustments were made for multiple testing.

Reverse transcriptase-PCR

RNA was extracted from the brains of MHV-infected and sham-infected mice at defined times post-infection (PI) using TRIzol® Reagent (Invitrogen, Carlsbad, CA), treated with RQ1 RNase-free DNase (Fisher Scientific, Pittsburgh, PA), and purified by phenol/chloroform extraction. cDNA was generated using an MMLV reverse transcriptase (RT) kit (Invitrogen) and random hexamer primers (Promega, Madison, WI). PCR was performed on the resulting cDNA with specific primers for GAPDH (forward, 5′-ACTCACGGCAAATTCAACG; reverse, 5′-CCCTGTTGCTGTAGCCGTA), IL-23p19 (forward, 5′-CATGGGGCTATCAGGGAGTA; reverse, 5′-AATAATGTGCCCCGTATCCA), and IL-12p35 (forward, 5′-GACTTGAAGATGTACCAGACAG; reverse, 5′-GAGATGAGATGTGATGGGAG). Amplification was performed on an Eppendorf MasterCycler (Westbury, NY) using the following profile: step 1, denaturation at 94°C for 45 sec; step 2, annealing at 60°C for 45 sec; and step 3, extension at 72°C for 1 min. Steps 1–3 were repeated 34 times for a total of 35 cycles and were followed by a 7-min incubation at 72°C. Sequence analysis of GAPDH, IL-23p19, and IL-12p35 amplicons confirmed primer specificity (54).

Virus and mice

MHV strain J2.2V-1 was used for the experiments described (58). Age-matched 5- to 7-week-old C57BL/6 mice (H-2^b background) were purchased from the National Cancer Institute, Bethesda, Maryland, and used for MHV experiments. Following anesthetization by IP injection with ketamine, mice were injected intracranially (IC) with virus (500 PFU) suspended in 30 μL of sterile saline. Experimental groups of mice infected with MHV were injected IP with either purified anti-IL-23p19 (CNTO 209, 500 μg/dose), anti-IL-12/23p40 (CNTO 3913, 500 μg/dose), or mouse IgG isotype control (CNTO 1322, 500 μg/dose) according to the following experimental schedules. For the acute MHV infection studies, mice were injected on days 0 and 7 PI with anti-IL-23p19 or control antibodies, and mice receiving anti-IL-12/23p40 or control antibodies were injected on days 0, 4, 8, and 12 PI. For the chronic MHV infection studies, mice were injected on days 14 and 21 PI with anti-IL-23p19 or control antibodies, and mice receiving anti-IL-12/23p40 or control antibodies were injected on days 12, 16, and 20 PI. Animals were sacrificed at defined time points and tissues removed for analysis. One-half of each brain at each time point was used for plaque assay on a mouse astrocytoma cell line (30). Experiments for all animal studies described have been reviewed and approved by an appropriate institutional review committee.

Flow cytometry

Mononuclear cells were obtained from half-brains and dCLN (deep and superficial nodes) at defined times post IC infection with MHV using previously described methods (23,30). Immunophenotyping of cells was performed using allophycoerythrin (APC)-conjugated rat anti-mouse CD4 and CD8 (Pharmingen, San Diego, CA), FITC-conjugated rat anti-mouse F4/80 (Serotec, Oxford, U.K.), and APC-conjugated rat anti-mouse CD45 (Pharmingen) (23). In all cases, isotype-matched control antibodies were used. Virus-specific $CD4^+$ and $CD8^+$ T cells recognizing their respective immunodominant epitope between amino acids 133 and 147 of the membrane (M) glycoprotein (M133–147) and surface (S) glycoprotein (S510–518) were determined by intracellular IFN-γ staining using previously described methods (14,16). In brief, 1×10^6 total cells were stimulated with 5 μM final concentration of viral peptides for 6 h at 37°C in media containing Golgi stop (Cytofix/Cytoperm kit; Pharmingen), after which cells were washed and blocked with PBS containing 10% FBS and a 1:200 dilution of rat anti-mouse CD 16/32 antibody CD16/32 (BD Pharmingen). Cells were then stained for surface antigens using APC-conjugated rat anti-mouse CD4 and CD8 (BD Pharmingen), according to the viral peptide stimulation condition, for 45 min at 4°C. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit and stained for intracellular IFN-γ using phycoerythrin (PE)-conjugated anti-IFN-γ (1:50; XMG1.2, BD Pharmingen) for 45 min at 4°C. Cells were washed and flow cytometry was performed using a FACStar flow cytometer (Becton Dickinson, Mountain View, CA). Frequency data are presented as the percentage of positive cells within the gated population. Total cell numbers were calculated by multiplying these values by the total number of live cells isolated.

Cytokine analysis

Supernatants from brain homogenates were analyzed for cytokine expression in a multiplexed fashion using the cytokine 20-plex AB bead kit (BioSource Invitrogen, Carlsbad, California). This kit comprises analyte-specific components for the measurement of mouse FGF basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL-13, IL-17, CXCL10, CXCL1, CCL2, CXCL9, CCL3, TNF-α, and VEGF. Samples were run in duplicate in accordance with the manufacturer's directions. Samples were analyzed using a Luminex 100 IS System (Austin, TX) running Star Station 2.0 software (Applied Cytometry Systems, Sacramento, CA). IL-23p19 was quantified using standard ELISA methods with rat anti-mouse IL-23p19 (CNTO 209) capture antibody, biotinlyated rat anti-mouse IL-12/23p40 (C17.8) antibody, and mIL-23 standard (R&D Systems).

Histology

Spinal cords were fixed in normal balance formalin for 24 h and then embedded in paraffin (30). Sections of spinal cords were stained with luxol fast blue to identify myelin (blue) and counterstained with Harrison hematoxylin and eosin to visualize cellular inflammation. Slides containing stained spinal cord sections were blinded and the severity of demyelination assessed using a light microscope. Demyelination was scored as follows: 0 = no demyelination; 1 = mild inflammation accompanied by loss of myelin integrity; 2 = moderate inflammation with increasing myelin damage; 3 = numerous inflammatory lesions accompanied by significant increase in myelin stripping; and 4 = intense areas of inflammation accompanied by numerous areas of phagocytic cells engulfing myelin debris (24,29,30).

Statistical analysis of MHV studies

Statistically significant differences between antibody treatment groups were determined by Mann-Whitney rank sum test. Statistical analyses were done using Sigma-Stat 2.0 software (Jandel, Chicago, IL) and p values of ≤ 0.05 were considered significant.

RESULTS

Anti-IL-23p19 mAb binds mouse IL-23 and neutralizes bioactivity

A monoclonal antibody specific for mouse IL-23p19 was developed as described in the materials and methods section. Mouse IL-12, IL-23, or p40 (shared by both IL-23 and IL-12) were coated on ELISA plates and anti-IL-23p19 binding determined. As shown in Fig. 1A, anti-IL-23p19 recognizes IL-23, but not either IL-12 or the p40 subunit, in a concentration-dependent manner indicating that this antibody is specific for mouse IL-23. To test the ability of anti-IL-23p19 to neutralize signaling, mouse splenocyte cultures were incubated with recombinant mouse IL-23 and IL-2, and IL-17A production determined. Inclusion of either anti-IL-23p19 or anti-IL-12/23p40 mAbs resulted in reduced IL-17A secretion by splenocytes and was sensitive to antibody concentration (Fig. 1B). Of note, CNTO 3913 and C17.8 anti-IL-12/23p40 mAbs demonstrated comparable neutralization potency against mouse IL-23 in this assay (data not shown). Incubation of cells with anti-IL-12p35 did not result in an appreciable decrease in IL-17A production. These data demonstrate that anti-IL-23p19 is specific for

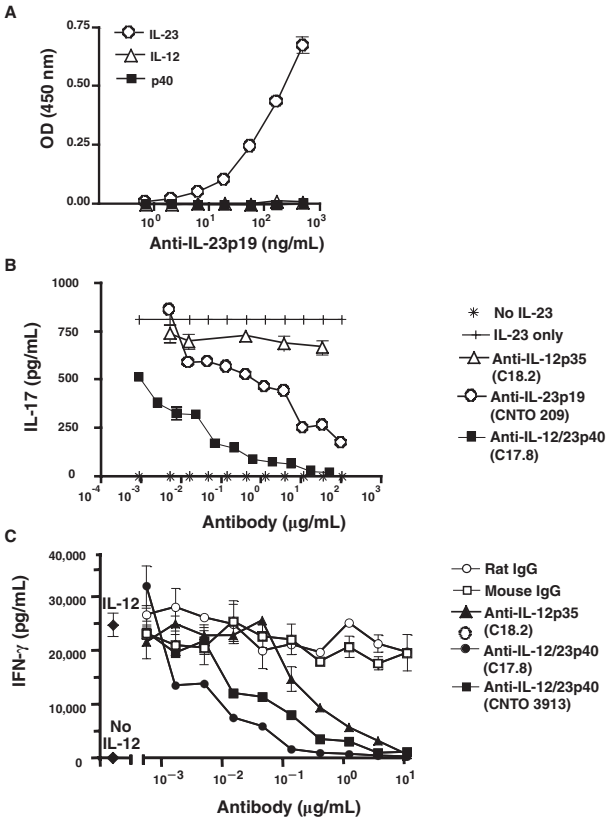


FIG. 1. Anti-IL-23 antibody is specific for mouse IL-23 and neutralizes IL-23 bioactivity. (**A**) Mouse IL-12, IL-23, or p40 homodimer were coated on ELISA plates and anti-IL-23 antibody was titrated. Data are shown as the mean optical density (OD) of replicate wells ± SD and are representative of two experiments. IL-17 or IFN-γ production was measured in supernatants from C57BL/6 splenocyte cultures supplemented with recombinant mouse (**B**) IL-23 or (**C**) IL-12 and dilutions of anti-IL-12/23p40, anti-IL-12p35, or anti-IL-23p19 antibodies. Data are shown as the mean of replicate wells ± SEM and are representative of three experiments.

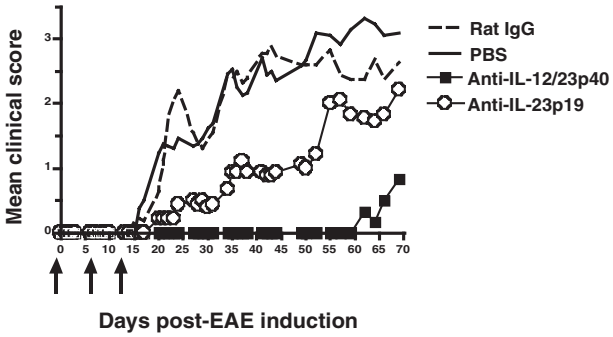


FIG. 2. IL-23 neutralizations inhibit MBP-induced EAE in B10.PL mice. EAE was induced in B10.PL mice as described in the materials and methods section, and animals were treated with PBS, negative control rat IgG, anti-IL-12/23p40, or anti-IL-23p19 mAbs at days –1, 6, and 13 (arrows). Daily clinical scores were averaged for the group and are shown through 70 d post–EAE immunization.

IL-23 and blocks signaling. In addition, the ability of anti-IL-12/23p40 blocking antibody to neutralize signaling was also determined. As shown in Fig. 1C, inclusion of either anti-IL-12p35 or anti-IL-12/23p40 antibodies resulted in a dose-dependent reduction in IFN-γ secretion by splenocytes incubated with recombinant IL-12. These data confirm that anti-IL-12/23p40 is capable of inhibiting IL-12–induced IFN-γ secretion by T cells. As expected, since the anti-IL-23p19 antibody does not bind mouse IL-12 (Fig. 1A), there was no neutralization of mouse IL-12 when anti-IL-23p19 was tested in this assay (data not shown).

We next determined the *in vivo* blocking efficacy of anti-IL-23p19 and anti-IL-12/23p40 antibodies. EAE is an autoimmune demyelinating disease that shares many clinical and histologic similarities with the human autoimmune demyelinating disease MS (5,12). Previous studies have demonstrated that IL-23p19–deficient mice are resistant to EAE and treatment of mice with a neutralizing anti-IL-23p19 antibody reduces disease severity (8). Blocking IL-23 signaling effectively limits specific pathways required for CNS autoimmune inflammation by inhibiting the generation of IL-17–producing T cells. To test the efficacy of the anti-IL-23p19-specific antibody for *in vivo* neutralization, chronic-relapsing EAE was induced in B10.PL mice by injecting MBP as described in the materials and methods section. Mice were treated with either anti-IL-23p19 or anti-IL-12/23p40 mAbs, control rat IgG, or PBS, and clinical disease progression in experimental groups was determined 70 d post-MBP immunization. Initiation of treatment with anti-IL-12/23p40 was more effective at suppressing mean daily clinical sign scores (Fig. 2), EAE incidence, the highest clinical score during acute EAE, and relapse number and severity (Table 1), when compared to anti-IL-23p19 treatment. However, both antibodies were able to significantly reduce mean daily clinical sign scores (Fig. 2), median time to onset, and disease burden over time when compared to mice treated with control antibody or PBS (Table 1). Anti-IL-23p19 and anti-IL-12/23p40 treatment also suppressed EAE severity when dosing was initiated on days 10 or 30 after EAE induction (data not shown). Collectively, these data demonstrate that both anti-IL-23p19 and anti-IL-12/23p40 antibodies are capable of specifically blocking cytokine signaling *in vivo* and reducing the severity of clinical disease associated with autoimmune-mediated neuroinflammation and demyelination.

IL-12 and IL-23 are not required for viral clearance from the brain

Utilizing primers specific for either IL-23p19 or IL-12p35, we tested for mRNA expression within the brain following IC MHV infection of the CNS (Fig. 3A). Expression of IL-12p35 was not detectable within the brains of sham-infected mice, but was clearly present at days 3, 7, and 14 PI. Transcripts for IL-23p19 are present in the brains of control mice and MHV infection results in an apparent increase in transcript levels at later times PI. Increased mRNA transcripts correlated with elevated lev-

TABLE 1. ADMINISTRATION OF ANTI-IL-23P19 AND ANTI-IL-12/23P40 REDUCES EAE CLINICAL DISEASE

Treatment regimen	*Incidence*	*Median time to onset (days)*	*Disease burden over time (mean ± SEM)*	*Highest acute clinical sign (mean ± SEM)*	*No. of relapses (mean ± SEM)*	*Relapse severity (mean ± SEM)*
Day −1 (start)						
PBS	13/13 (100%)	22.5	1.66 ± 0.20	3.6 ± 0.3	0.7 ± 0.2	3.8 ± 0.1
Rat IgG	11/11 (100%)	21.5	1.27 ± 0.20	3.8 ± 0.4	0.5 ± 0.2	3.5 ± 0.3
Anti-IL-12/23p40	3/6 (50%)[a,b]	>69[a,b]	0.18 ± 0.24[a,b]	0.8 ± 0.5[a,b]	0.0 ± 0.0	0.0 ± 0.0
Anti-IL-23p19	8/9 (89%)	51.0[a,b]	0.80 ± 0.24[a]	2.8 ± 0.5	0.4 ± 0.2	3.3 ± 0.3

[a]Significantly different from PBS at $p < 0.05$.
[b]Significantly different from rat IgG at $p < 0.05$.

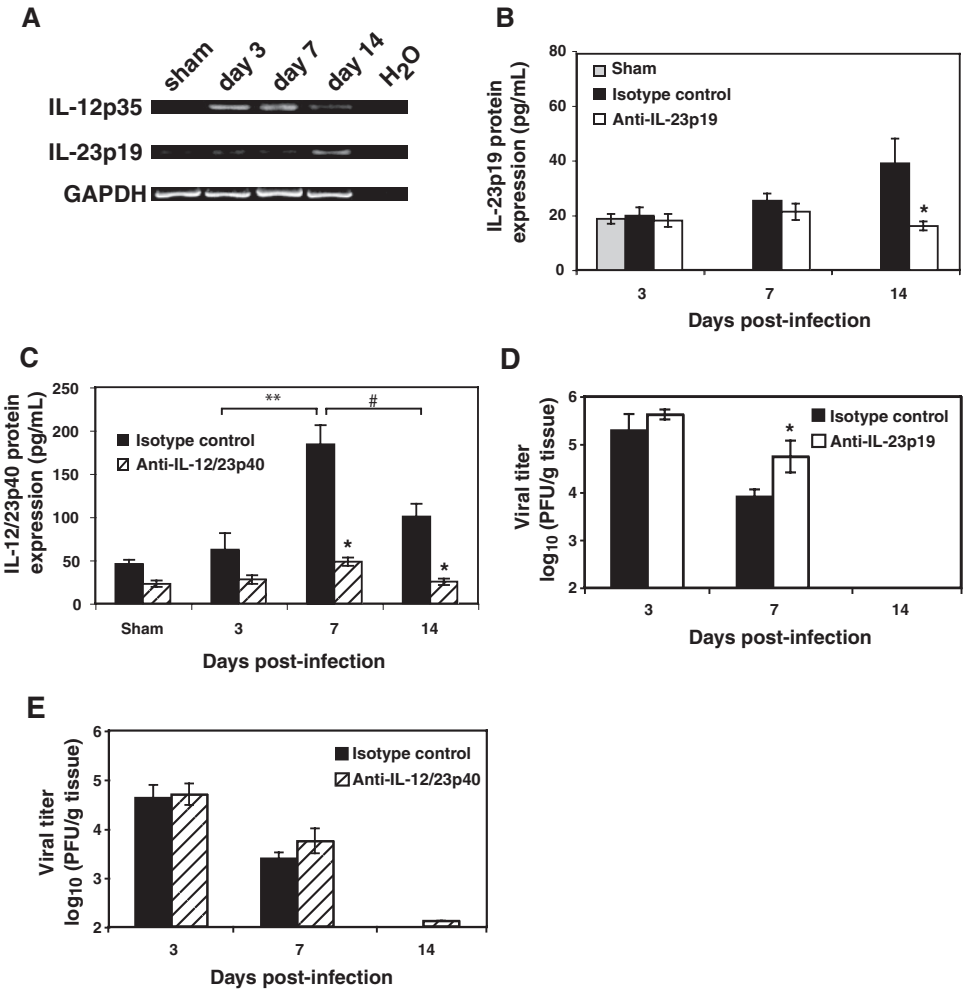


FIG. 3. Antibody blocking of either IL-23p19 or IL-12/23p40 does not affect the ability to control viral replication within the brain. (**A**) RT-PCR revealed that intracranial instillation of MHV results in detection of transcripts specific for IL-23p19 and IL-12p35 in the brain at the indicated times post-infection. Each lane is from the brain of a single mouse and is representative of n = 2–3 for each time point. ELISA indicating that both IL-23p19 (**B**) and IL-12/23p40 (**C**) are detected within the brains of MHV-infected mice at the defined time points post-infection (n = 3–5 mice for each time point). Levels of IL-12/23p40 within the isotype control-treated mice were significantly elevated within the brain at day 7 post-infection when compared to levels present at day 3 (**$p \leq 0.007$) and day 14 (#$p \leq 0.03$) post-infection. Neutralizing antibody treatment significantly reduced ($p \leq 0.05$) cytokine protein levels during peak expression in the brain compared to isotype control treatments at the indicated times post-infection. Treatment of MHV-infected mice with neutralizing antibodies specific for either IL-23p19 (**D**) or IL-12/23p40 (**E**) does not impair control of viral replication (PFU/g tissue) from the CNS. Data are presented as average ± SEM and represent a minimum of two independent experiments with a total n = 5–9 for each antibody treatment group per time point examined. *$p \leq 0.05$ compared to mice treated with control antibody.

els of IL-23p19 and IL-12/23p40 (Fig. 3B and C) within the brains of infected mice as determined by ELISA, although IL-23p19 was present at markedly reduced levels compared to levels of IL-12/23p40 between 7 and 14 days PI. Levels of IL-12/23p40 were significantly elevated within the brains of mice at day 7 PI when compared to either day 3 ($p \leq 0.007$) or day 14 ($p \leq 0.03$) PI (Fig. 3C). We next tested whether blocking either IL-12 or IL-23 affected antiviral immune responses following MHV infection of the CNS. C57BL/6 mice were infected IC with MHV and treated IP with either anti-IL-23p19 or anti-IL-12/23p40 mAbs or control IgG, and viral clearance from the brains determined. *In vivo* treatment with neutralizing anti-IL-23p19 or anti-IL-12/23p40 antibodies significantly reduced their respective cytokine protein levels in the brains of MHV-infected mice, compared to control treatment groups (Fig. 3B and C). Viral titers were elevated ($p \leq 0.05$) within the brains of mice treated with

anti-IL-23p19 (Fig. 3D) at day 7 PI compared to mice treated with the isotype control antibody, suggesting an impaired ability to control viral replication. However, there were no differences in viral titers within the brains of mice treated with anti-IL-12/23p40 (Fig. 3E), compared to mice treated with control antibody at any time point examined. Ultimately, both groups of mice reduced viral titers to levels comparable to control mice at day 14 PI (~100 PFU/g tissue). These data indicate that neither IL-12 nor IL-23 signaling is required for viral clearance from the brains of MHV-infected mice. In order to further evaluate host defense to MHV infection, we used flow cytometric phenotyping to determine T-cell responses in mice treated with either anti-IL-23p19 or anti-IL-12/23p40 blocking antibody. There were no significant differences in total $CD4^+$ or $CD8^+$ T-cell accumulation in the brains of mice treated with either anti-IL-23p19 (Fig. 4A) or anti-12/23p40 (Fig. 4B) at any time point examined. Moreover, there were no differences in frequencies of virus-specific IFN-γ–producing $CD4^+$ or $CD8^+$ T cells (as determined by intracellular IFN-γ staining in response to defined immunogenic viral peptides) within the brains of MHV-infected mice in which either IL-23 (Fig. 4C) or IL-12/23 (Fig. 4D) signaling was blocked when compared to control-treated mice at days 7 and 14 PI.

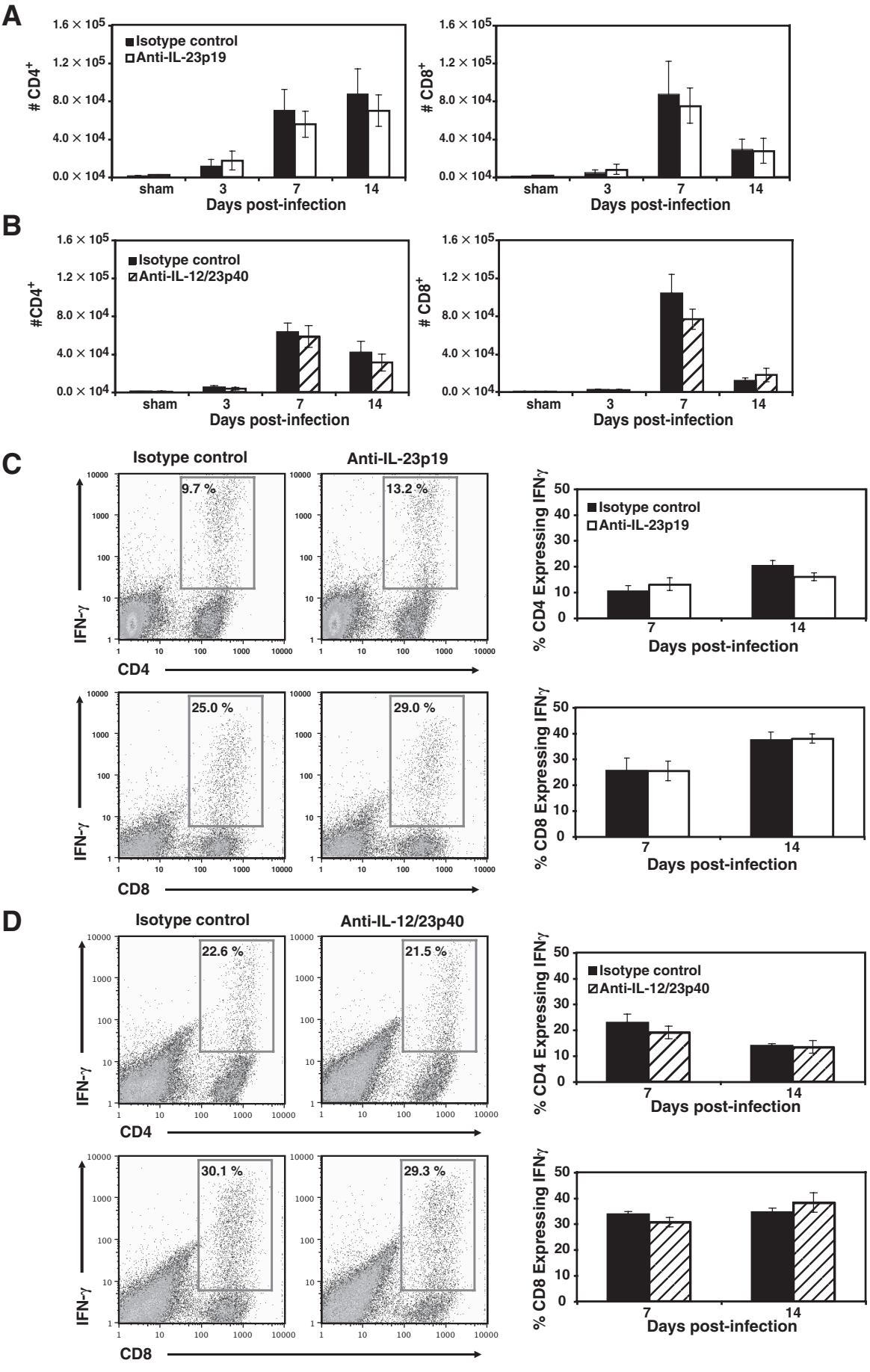
Antiviral T-cell effector functions and IL-23 signaling

Infiltrating virus-specific T cells eliminate MHV from the brain through secretion of IFN-γ by both $CD4^+$ and $CD8^+$ T cells and cytolytic activity by CD8+ T cells (2,48,55). Although viral clearance from the brains of mice treated with either anti-IL-23p19 or IL-12/23p40 was not affected, treatment with anti-IL-23p19 antibody did result in significantly higher viral titers at day 7 PI compared to mice treated with control antibody, suggesting muted antiviral functional activity by infiltrating T cells. Therefore, to determine if antibody treatment affected antiviral effector functions associated with virus-specific T cells, cytokine profiles within the brains of experimental mice were determined by ELISA. There was no difference in expression of IFN-γ within the brains of mice treated with anti-IL-23p19 compared to animals treated with control antibody at any time point examined (Fig. 5A). On day 3 PI the levels of IFN-γ secretion in the brains of MHV-infected mice were slightly increased compared to sham-infected mice. However, a dramatic increase in IFN-γ secretion (~30 pg/mL in experimental mice) was observed in both infected treatment groups on day 7 PI, correlating with infiltration of virus-specific T cells into the CNS (39). By day 14, viral titers in the brain were reduced and IFN-γ levels in anti-IL-23p19 and control antibody-treated mice decreased appreciably (Fig. 5A). Expression profiles of TNF-α at days 3, 7, and 14 follow a similar trajectory as IFN-γ between experimental groups (Fig. 5B). However, at day 3 PI, TNF-α levels were elevated within the brains of anti-IL-23p19–treated mice compared to control-antibody-treated mice, yet this difference was not significant. However, there were no differences in TNF-α levels at either days 7 or 14 between anti-IL-23p19–treated or control mice (Fig. 5B). Notably, TNF-α levels were markedly reduced within the brain compared to IFN-γ levels, supporting earlier studies indicating that MHV infection effectively reduces synthesis of TNF-α protein (56). Blocking IL-23 did not significantly change the expression of IL-4 levels in the brain compared to control-treated mice, although there was a slight reduction on day 14 PI (Fig. 5C). Similarly, MHV infection did not result in increased IL-17 expression in the brain, and blocking IL-23 expression had no effect compared to control mice at any time point examined (Fig. 5D). There were no differences in production of detectable levels of other cytokines or chemokines measured within the brains of mice treated with either anti-IL-23p19 or control antibody (data not shown). Finally, there were no differences in cytolytic activity of CNS-infiltrating $CD8^+$ T cells between anti-IL-23p19– and control antibody–treated mice, indicating that IL-23 does not influence CTL activity in response to MHV infection (data not shown).

Anti-IL-23p19 does not reduce demyelinating disease in persistently infected mice

Mice were infected IC with MHV and antibody treatment was initiated between days 12 and 14, which rep-

FIG. 4. Blocking IL-12/23 does not alter T-cell infiltration into the CNS of MHV-infected mice. MHV-infected mice were treated with either anti-IL-23p19 or anti-12/23p40 neutralizing antibodies and the effects on T-cell infiltration into the CNS evaluated at specific time points post-infection. Blocking either IL-23 (**A**) or IL-12/23 (**B**) did not reduce total numbers of $CD4^+$ or $CD8^+$ T cells entering the CNS when compared to mice treated with control antibody. Similarly, treatment with anti-IL-23p19 (**C**) or anti-IL-12/23p40 (**D**) did not alter infiltration of virus-specific $CD4^+$ or $CD8^+$ T cells compared to control-antibody-treated mice, as shown in representative FACS dot-plots and corresponding bar graphs. The bar graphs portray the frequency of virus-specific $CD4^+$ or $CD8^+$ T cells within their respective T-cell subset on days 7 and 14 post-infection. The frequencies of virus-specific T cells were derived from dual-positive IFN-γ^+ $CD4^+$/$CD8^+$ populations as shown in boxed regions of representative dot-plots from day 7 post-infection. Data are presented as average ± SEM and are representative of two independent experiments with a total n = 4–8 for each antibody treatment group per time point examined.


A
CD4+
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Isotype control
Anti-IL-23p19
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3
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Days post-infection
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C
Isotype control
Anti-IL-23p19
9.7 %
13.2 %
IFN-γ
CD4
25.0 %
29.0 %
CD8
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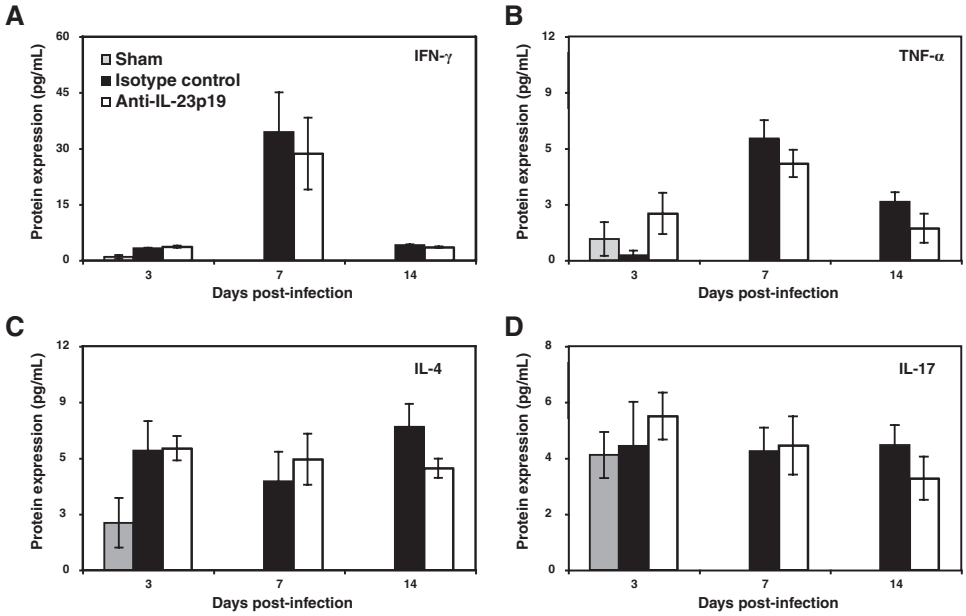

FIG. 5. Anti-IL-23p19 treatment does not modulate cytokine expression in the CNS of MHV-infected mice. The temporal expression of cytokines was determined by ELISA in sham-infected and MHV-infected mice treated with either anti-IL-23p19 or control antibody. Cytokines examined include IFN-γ (**A**), TNF-α (**B**), IL-4 (**C**), and IL-17 (**D**). Data are presented as average ± SEM and represent two independent experiments with a total n = 4 for each antibody treatment group per time point examined.

resents a point at which demyelination is established (36). There were no differences in clinical disease progression between mice treated with either anti-IL-12/23p40 or IL-23p19 when compared to mice treated with control antibody (data not shown). Analysis of spinal cords from mice at days 21 and 28 indicated that anti-IL-23p19 or anti-IL-12/23p40 treatment did not reduce the severity of white matter damage or demyelination as compared to control mice (Fig. 6A, B, C, and D). Accordingly, immunophenotyping the composition of the cellular infiltrate within the brains and spinal cords indicated similar numbers and frequency of T cells and macrophages when anti-IL-23p19– or anti-IL-12p40–treated mice were compared to control-treated mice on days 21 and 28 PI (data not shown).

DISCUSSION

The present study was undertaken to investigate IL-12 and IL-23 signaling mechanisms associated with a protective immune response following viral infection of the CNS using the MHV model of viral-induced encephalomyelitis. In addition, the functional role of IL-12 and IL-23 in contributing to demyelination in MHV-infected mice was also determined. Previous studies have demonstrated that MHV infection of either IFN-$\gamma^{-/-}$ mice or IFN-γR$^{-/-}$ mice results in increased mortality accompanied by increased viral titers within infected tissues, supporting the importance of generating a Th-1 response in host defense (17,48,51,52). However, the signaling mechanisms promoting the development of a Th-1 response in MHV-infected mice remain elusive. Parra *et al*. (47) demonstrated increased expression of numerous cytokines, including IL-12/23p40, within the brains following either lethal or non-lethal coronavirus-induced acute encephalomyelitis, indicating that IL-12 expression may influence the generation of IFN-γ–secreting T cells. However, MHV infection of IL-12–deficient mice (lacking either the p35 or p40 subunit) were able to clear virus from the liver in an IFN-γ–dependent manner, indicating that IL-12 signaling is not essential for protection (51,52). Using antibodies specific for IL-12/23 or IL-23, the work presented here supports previous findings and demonstrates that neither cytokine is necessary for generating functional MHV-specific T cells in response to CNS infection or trafficking of virus-specific T cells into the CNS. In addition, antibody-mediated neutralization of IL-23 had no effect on the secretion of IFN-γ. Although viral titers were eventually reduced below levels of detection in mice treated with blocking IL-23p19 antibody by day 14 PI, there was a significant increase in viral titers within the brains at day 7 PI in anti-IL-23p19–treated mice, suggesting that IL-23 signaling may have some role in controlling viral replication. Clearly, this is not an effect on T-cell antiviral function, as inhibition of IL-23

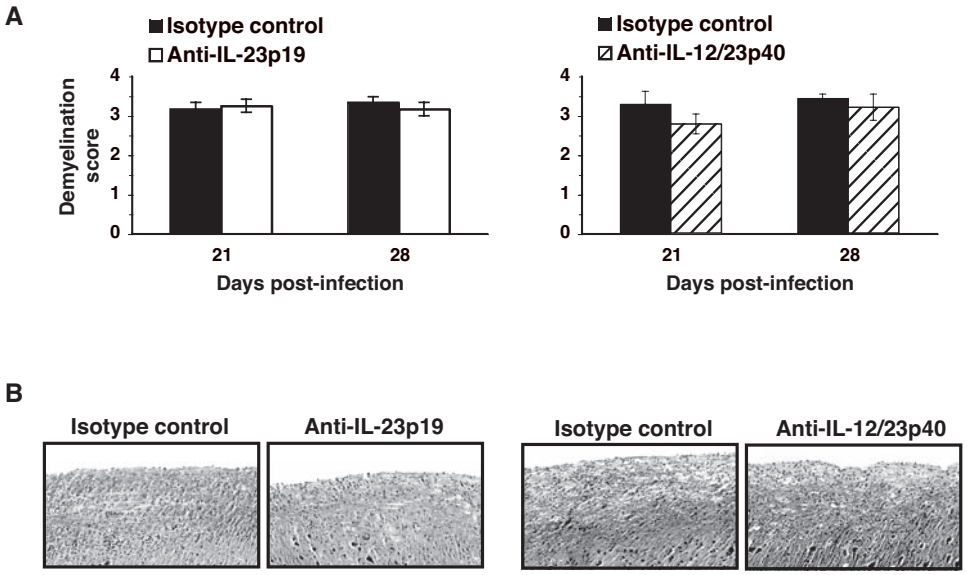

FIG. 6. Chronic MHV-induced demyelinating disease is not affected following anti-IL-23p19 or anti-IL-12/23p40 treatment. Histologic scoring of the severity of white matter damage to the spinal cords of mice from anti-IL-23p19 (**A**) and anti-IL-12/23p40 (**B**) treatment groups indicated no differences in disease severity at either day 21 or 28 post-infection compared to control treated mice. Data were derived from a minimum of three mice per antibody treatment group for each time point, and represent at least two independent experiments. Representative luxol fast blue staining of spinal cords (×100) revealed no difference in the severity of demyelination in anti-IL-23p19 (**A**) and anti-IL-12/23p40 (**B**) treated mice when compared to control treated mice at day 21 post-infection. Data are presented as average ± SEM.

signaling did not affect either cytokine secretion or CTL activity of infiltrating virus-specific T cells. However, it is possible that IL-23 may influence as of yet undefined antiviral effector responses by resident cells of the CNS that participate in host defense during acute disease. Administration of anti-IL-23p19 or anti-IL-12/23p40 antibody in mice persistently infected with virus did not affect either T-cell or macrophage accumulation in the CNS, or lessen the severity of demyelination, indicating that IL-23 and IL-12 signaling is not important during chronic demyelinating disease in mice persistently infected with MHV. Our data highlight the differences between IL-23 in a model in which autoreactive lymphocytes are not thought to participate in disease (i.e., MHV infection) and EAE, which is characterized by infiltration and accumulation of autoreactive T cells that are essential in driving demyelination. Importantly, we feel that IC infection, while not a natural route of infection, does not invalidate conclusions derived from this study, as this method of infection reproducibly results in an acute encephalomyelitis followed by a chronic demyelinating disease. Moreover, this method of infection results in generation of virus-specific lymphocytes within peripheral lymphatic tissue that occurs following peripheral administration of virus (40).

Instillation of MHV into the CNS resulted in detectable mRNA transcripts specific for IL-12p35 and IL-23p19 within the brain following viral infection. Although the cellular source for these transcripts was not determined, it is likely that both astrocytes and microglia produce IL-12 and IL-23 in response to MHV infection, as previous studies have revealed that these cells are capable of expressing these cytokines both *in vivo* and *in vitro* (6,32,34). These findings indicate that genes responsible for encoding IL-12 and IL-23 contain promoter elements sensitive to factors released in response to MHV infection, and that preferential expression of either IL-12 or IL-23 does not occur. This observation is not unique, as both gram-positive and gram-negative bacteria can elicit production of both IL-12 and IL-23 (4). Moreover, specific agonists for toll-like receptors including CpG and LPS also stimulate IL-12 and IL-23 expression (49,57). Although MHV infection promotes expression of IL-12 and IL-23 within the brain as well as in secondary lymphatic tissue (data not shown), a distinct Th-1 lineage is developed, as IFN-γ is a predominant cytokine expressed by virus-specific T cells, and Th-2 cytokines such as IL-4 are present at comparatively lower levels within both brain and dCLN early following infection. In marked contrast, IL-17 protein is not detectable by ELISA within dCLN (data not shown), and is present at extremely low levels ($\leq$10 pg/mL) within the brains of infected mice. Therefore, the lineage fate of T cells must be decided very early following infection, allowing for MHV-spe-

cific T cells to generate a predominant Th-1 response for optimal protection. What remains to be determined is identifying the key signaling events that regulate T-cell differentiation in response to MHV infection, as our findings suggest that neither IL-23 nor IL-12 is essential in this process. Presumably, soluble elements provided by antigen-presenting cells are necessary in tailoring T-cell commitment to a specific lineage. For instance, IL-18 production by antigen-presenting cells can work synergistically with IL-12 to facilitate IFN-γ induction (43). However, this stimulation may not be prominent for viral pathogens, as pulmonary adenovirus infection studies reveal a Th-1 response is still mounted in the absence of both IL-12 and IL-18, despite diminished IFN-γ production (64). Furthermore, the role of TCR signaling strength in relation to IFN-γ induction adds to the complexity of Th-1 commitment (43). Whether the IL-12–independent Th-1 response observed in MHV infection is mediated by TCR antigen-signaling strength or by regulating key T-cell transcription factors such as STAT1 that are necessary for IFN-γ expression, remains to be determined (11,43).

While the functional role of IL-23 in initiating and amplifying autoimmune diseases has been well documented, how IL-23 may regulate immune responses following microbial infection has been less well characterized. Infection of IL-23–deficient mice with *Toxoplasma* revealed moderate resistance to infection, suggesting that IL-23 is not critical in mounting a protective immune response (35). Respiratory syncytial virus infection of STAT1-deficient animals results in elevated levels of IL-17 that correlated with increased IL-23p19 expression and was accompanied by increased viral burden within the lungs (21). These findings reveal that the absence of the transcription regulator STAT1 amplifies IL-17, yet does not enhance antiviral effector responses by T cells. Vesicular stomatitis virus infection of mice lacking IL-12 receptor beta 1 (IL-12βR1), a shared receptor for IL-12 and IL-23, did not modulate an effective immune response against the virus, suggesting that IL-12/IL-23 are not essential in recovery from viral encephalitis (25). In contrast, infection of mice deficient in IL-12p40, IL-23p19, or IL-17 with *Klebsiella pneumoniae* resulted in increased mortality, highlighting critical roles for IL-23 and Th-17 cells in pulmonary host defense (18,20). Transient delivery of IL-23 controls growth of *Mycobacterium tuberculosis* within the lungs of mice that was associated with augmented T-cell responses (19).

CONCLUSION

Clearly, the dependence on IL-23/IL-17 in providing host defense following infection is controlled by various factors and most likely is dictated by innate immune responses. However, the findings presented in this study provide clear distinction between the functional roles of IL-12/23 signaling pathways in the development of autoimmune inflammatory diseases versus protective antiviral T-cell responses. Importantly, the data presented in this paper suggest that therapeutic strategies designed to inhibit IL-12 and/or IL-23 for treatment of various human autoimmune diseases may not significantly impair the generation of functional virus-specific T cells (26,28,38).

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