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Possible role of interleukin-17 in a prime/challenge model of multiple sclerosis

Jane E. Libbey · Ikuo Tsunoda · Robert S. Fujinami

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Abstract No one single pathogen has been identified as the causative agent of multiple sclerosis (MS). Alternately, the likelihood of an autoimmune event may be nonspecifically enhanced by different infectious agents. In a novel animal model of MS, SJL/J mice primed through infection with a recombinant vaccinia virus (VV) encoding myelin proteolipid protein (PLP) (VV_{PLP}) were susceptible to a central nervous system (CNS) inflammatory disease following administration of a nonspecific immunostimulant [complete Freund's adjuvant (CFA) plus *Bordetella pertussis* (*BP*)]. Mononuclear cells isolated from the brains, but not the spleens, of VV_{PLP}-primed CFA/*BP* challenged mice produced interleukin (IL)-17 and interferon-γ and transferred a CNS inflammatory disease to naïve SJL/J mice. Administration of curdlan, a T helper 17 cell inducer, unexpectedly resulted in less severe clinical and histological signs of disease, compared to CFA/*BP* challenged mice, despite the induction of IL-17 in the periphery. Further examination of the VV_{PLP}-prime CFA/*BP* challenge model may suggest new mechanisms for how different pathogens associated with MS can protect or enhance disease.

J. E. Libbey · R. S. Fujinami (✉)
Department of Pathology, University of Utah School of Medicine,
30 North 1900 East, 3R330 SOM,
Salt Lake City, UT 84132, USA
e-mail: Robert.Fujinami@hsc.utah.edu

I. Tsunoda
Department of Microbiology and Immunology,
Louisiana State University Health Sciences Center,
1501 Kings Highway,
Shreveport, LA 71130, USA

I. Tsunoda
Center for Molecular & Tumor Virology,
Louisiana State University Health Sciences Center,
1501 Kings Highway,
Shreveport, LA 71130, USA

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Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating, autoimmune disease of the central nervous system (CNS) (Compston and Coles 2008). The etiology of MS is currently unknown; however, microbial infections have been shown to play a role in disease exacerbations [(Kriesel and Sibley 2005), reviewed in (Libbey and Fujinami 2010)]. Despite extensive investigation, no single causative pathogen has been identified for MS. A proposed alternate hypothesis is that many infectious agents nonspecifically enhance the likelihood of an autoimmune event (Tsunoda et al. 2007; von Herrath et al. 2003). This hypothesis would require a two-step process. The first "priming" step would involve the *de novo* activation of autoreactive T cells through molecular mimicry. Molecular mimicry is cross-reactivity between self and microbial epitopes during a microbial infection (Cusick et al. 2012; Fujinami et al. 1983; Fujinami and Oldstone 1985; Libbey et al. 2007). The second "challenge" step would involve the nonspecific activation of pre-existing autoreactive T cells through bystander activation. Bystander activation is caused by inflammatory responses, such as the production of cytokines, elicited by nonspecific immunostimulants or irrelevant infections (McCoy et al. 2006).

Previously, our group demonstrated that SJL/J mice primed through infection with a recombinant vaccinia virus (VV) encoding myelin proteolipid protein (PLP) (VV_{PLP}) were susceptible to the development of a CNS inflammatory disease following administration of a nonspecific immunostimulant

consisting of complete Freund's adjuvant (CFA) plus *Bordetella pertussis* (*BP*) cells (Theil et al. 2001; Tsunoda et al. 2007). The VV_{PLP} construct, which is a virus encoding a self protein, mirrors a molecular mimic, which is a virus encoding an immunologically cross-reactive epitope, and infection with this construct alone does not lead to the development of overt clinical or histological autoimmune disease (Theil et al. 2001). Secondary challenge of these "primed" mice with CFA/*BP* resulted in clinically and histologically evident CNS inflammatory disease, suggesting that bystander activation can function on antigen-specific cells originally induced by molecular mimicry (Theil et al. 2001; Tsunoda et al. 2007).

Two common animal models of MS are: Theiler's murine encephalomyelitis virus (TMEV) infection, which is a viral model, and experimental autoimmune encephalomyelitis (EAE), which is an autoimmune disease model [reviewed in (Tsunoda and Fujinami 1996)]. The VV_{PLP}-prime CFA/*BP* challenge model, which mirrors a molecular mimic, is similar to the TMEV infection model in that both involve a live viral infection, and the clinical signs induced by this prime/challenge model more closely resemble clinical signs induced by TMEV (impaired righting reflex) than typical EAE signs of disease. Molecular mimicry has been shown to play a pathogenic role in TMEV infection, where anti-TMEV serum and antibody against TMEV capsid protein cross-react with a myelin component, contributing to disease exacerbation [reviewed in (Tsunoda and Fujinami 1999)]. The VV_{PLP}-prime CFA/*BP* challenge model is also similar to the EAE model in that the VV construct encodes PLP and EAE can be induced through inoculation with CNS proteins, to include PLP, emulsified in CFA [reviewed in (Tsunoda and Fujinami 1996)].

MS is thought to be driven by both the T helper (Th) 1 and Th17 subsets of $CD4^+$ T cells [reviewed in (Chen et al. 2012; Jadidi-Niaragh and Mirshafiey 2011)]. Likewise, the disease induced by TMEV infection appears to be partly dependent on $CD4^+$ T cells [reviewed in (Tsunoda and Fujinami 1996)], and both the Th1 and Th17 subsets have been implicated in disease development following TMEV infection of susceptible mouse strains (Hou et al. 2009; Pullen et al. 1994). Increasing the Th1 response, by injecting interferon (IFN)-γ into the brain, enhanced the development of disease following TMEV infection (Pullen et al. 1994), and Th17 cells and the cytokine they produce, interleukin (IL)-17, were demonstrated to be induced and to play an immunomodulatory role in TMEV infection (Hou et al. 2009). EAE is also predominantly mediated by autoreactive CNS specific $CD4^+$ T cells in that, in general, $CD4^+$ Th1 cells and Th1-type cytokines [IL-2, lymphotoxin, IFN-γ] promote EAE, and until recently $CD4^+$ Th1 cells were thought to be the main effector cells responsible for the disease [reviewed in (Tsunoda and Fujinami 1996)]. However, Th17 cells are also effector cells in EAE (Harrington et al. 2005). The relative role of the Th1 or Th17 cell types in the pathogenesis of disease is still under investigation [reviewed in (Fletcher et al. 2010)].

Two related family members of IL-17, IL-17A and IL-17F, are produced by Th17 cells and Th17 cells also produce the proinflammatory cytokines IL-21, IL-22, IL-26 and tumor necrosis factor (TNF)-α [reviewed in (Jadidi-Niaragh and Mirshafiey 2011)]. Curdlan, a Th17 cell inducer, is an extracellular polysaccharide produce by *Agrobacterium* species [reviewed in (Laroche and Michaud 2007; McIntosh et al. 2005)]. Antigen-presenting cells, macrophages and dendritic cells, which express the C-type lectin, dectin-1, on their cell surface, can be activated by curdlan (LeibundGut-Landmann et al. 2007; Yoshitomi et al. 2005). Curdlan-induced dectin-1 signaling promotes dendritic cell maturation and secretion of proinflammatory cytokines. In turn, activated dendritic cells and proinflammatory cytokines are involved in the differentiation of the IL-17-producing subset of $CD4^+$ effector T cells (Th17 cells) (LeibundGut-Landmann et al. 2007; Yoshitomi et al. 2005).

In the current study, the ability of mononuclear cells (MNCs), isolated from the brains, but not the spleens, of mice primed with VV_{PLP} and challenged with CFA/*BP*, to transfer a CNS inflammatory disease to naïve SJL/J mice was demonstrated. In addition, a possible role for IL-17 in transfer of disease was observed. Finally, the ability of curdlan, a Th17 cell inducer, to modulate the resultant CNS inflammatory disease was examined. Our initial hypothesis was that curdlan would exacerbate the CNS inflammatory disease, since curdlan induces Th17 cells and Th17 cells function as effector cells in MS, TMEV and EAE disease; however, curdlan was found to ameliorate both the clinical and histological signs of disease despite the induction of IL-17 in the periphery.

Materials and methods

Animal experiments

Four- to 6-week-old female SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The care and use of the mice were in accordance with the guidelines prepared by the committee on Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council.

Mice were infected intraperitoneally (i.p.) with 1×10^6 plaque forming units of VV_{PLP} (encoding rat PLP) in 200 μl phosphate-buffered saline (PBS) (Barnett et al. 1993). Five weeks after VV_{PLP} infection, mice were injected subcutaneously (s.c.) at the base of the tail with 200 μl of CFA, which was a mixture of equal volumes PBS and Imject® Freund's incomplete adjuvant (IFA, Pierce

Biotechnology, Rockford, IL) containing 2 mg/ml *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI). Mice also received two intravenous injections (on the same day as the CFA injection and on the following day) of 5×10^9 *BP* cells (Michigan Department of Public Health, Lansing, MI) in 100 μl PBS. Mice were monitored for clinical signs. Three weeks after CFA/*BP* challenge mice were killed. The tissues from some of these mice were harvested for histology (see below). The brains and spleens from others of these mice were harvested and MNCs were isolated with Percoll™ (GE Healthcare Bio-Sciences, Piscataway, NJ). MNCs isolated from the brains of three mice were pooled. The MNCs were stimulated with concanavalin A (Con A, Sigma-Aldrich, St. Louis, MO), expanded with the Dynabeads® Mouse CD3/CD28 T Cell Expander (Invitrogen, San Diego, CA), as per the manufacturer's recommendation, in the presence of IL-2 (10 U/ml, BD Pharmingen, San Jose, CA), or not treated for 3 weeks in vitro. Supernatant was saved for enzyme-linked immunosorbent assays (ELISAs) (see below), and MNCs (1×10^6 or 1×10^7 cells in 200 μl RPMI media per mouse) from the CD3/CD28 expanded cultures were transferred i.p. into naïve SJL/J mice. Mice were monitored for clinical signs and killed 3 weeks after transfer.

Additional mice were infected with VV_{PLP}, as described above. Five weeks after VV_{PLP} infection, mice were injected s.c. with 200 μl of curdlan (5 mg/ml in PBS, Wako Chemicals, Richmond, VA) mixed with an equal volume of IFA. Mice were monitored for clinical signs and killed at 2–3 weeks post curdlan/IFA challenge.

Additional mice were infected with VV_{PLP}, as described above. Five weeks after VV_{PLP} infection, mice were injected i.p. with 200 μl curdlan (25 mg/ml) in PBS alone. Mice were monitored for clinical signs and killed at 2 weeks post curdlan/PBS challenge.

Clinical signs of disease in mice were evaluated through weight change and by an impaired righting reflex test, which are commonly used to evaluate disease in the TMEV inflammatory disease model of MS (Rauch et al. 1987; Tsunoda et al. 2001). When the proximal end of the mouse's tail is grasped and twisted to the right and then to the left, a healthy mouse resists being turned over (score of 0). If the mouse is flipped onto its back but immediately rights itself on one side or both sides, it is given a score of 1 or 1.5, respectively. If it rights itself in 1 to 5 s, the score is 2. If righting takes more than 5 s, the score is 3.

Histology

Mice were euthanized with isoflurane at the time points indicated above. Animals were perfused with PBS followed by 4% paraformaldehyde. Harvested brains were divided coronally into five portions and spinal cords were divided transversely into 12 portions. Tissues were embedded in paraffin. Four-micrometer-thick tissue sections were cut, mounted on slides and stained with Luxol fast blue. Neuropathology was scored, in a blinded fashion, as previously described, with slight modification as follows: 0, no lesions; 1, meningeal thickening, or MNC and/or polymorphonuclear cell collection in the meninges, or a small number of T cells detected in the meninges by immunohistochemistry against CD3; 2, perivascular cuffing in the parenchyma and/or meninges (Tsunoda et al. 1998, 2000, 2007). T cells were visualized on serial paraffin sections by the avidin–biotin peroxidase complex technique, using anti-CD3ε antibody (following trypsinization, 1:30 dilution, DakoCytomation, Carpinteria, CA), as previously described (Tsunoda et al. 2007).

Serum and supernatant ELISAs

Mice were bled when killed. The concentration of IL-17A was measured, in duplicate, in mouse sera and MNC tissue culture supernatants (see above) using the Mouse IL-17A ELISA MAX™ *Deluxe* Set (Biolegend, San Diego, CA) according to the manufacturer's recommendations. The concentration of IFN-γ was measured, in duplicate, in MNC tissue culture supernatants using the BD OptEIA™ Mouse IFN-γ ELISA Set (BD Pharmingen) according to the manufacturer's recommendation.

Statistical analysis

The StatView program (SAS Institute Inc., Cary, NC) was used for all statistical analyses performed. Analysis of variance (ANOVA), followed when indicated by the Fisher's Protected Least Significant Difference (Fisher's PLSD) post hoc test, was used to determine group differences for continuous data (weight, IL-17 ELISA). The unpaired two-group Mann–Whitney *U* test was performed for all nonparametric analyses (righting reflex score, neuropathology score).

Results

Transfer of disease, via MNC transfer, from VV_{PLP}-primed CFA/BP challenged mice to naïve mice

Previously, we demonstrated that administration of the non-specific immunostimulant, CFA/*BP*, induced disease in SJL/J mice subclinically primed with VV_{PLP} (Theil et al. 2001; Tsunoda et al. 2007). Here, we tested the ability of MNCs, isolated from the brains and spleens of these mice, to transfer disease. Isolated MNCs were expanded in vitro in the presence of CD3/CD28 beads prior to transfer. The mice

received either 1×10^7 expanded spleen MNCs alone or 1×10^6 expanded brain MNCs in addition to the 1×10^7 expanded spleen MNCs. The recipient mice were weighed and scored for righting reflex, both clinical signs of disease, daily for 3 weeks, at which point the animals were killed and brains and spinal cords were processed and scored for neuropathology. Over the course of the 3 weeks post MNC transfer, clinical signs of disease were not readily evident. Differences in weight change, between the mice receiving brain MNCs and the mice receiving spleen MNCs alone, were not large and only reached significance on 4 days, 3 days on which the mice receiving brain MNCs had a greater weight gain [days2 and 4 ($p<0.05$, ANOVA) and day9 ($p<0.01$, ANOVA)] and 1 day on which the mice receiving the spleen MNCs alone had a greater weight gain [day17 ($p<0.05$, ANOVA)] (Fig. 1a). The righting reflex scores were low overall and although the mice receiving brain MNCs tended to have higher scores than the mice receiving spleen MNCs alone, the differences were not significant (Fig. 1b, $p>0.05$, Mann–Whitney *U*). Upon examination of the neuropathology, however, it was found that the mice receiving brain MNCs had higher neuropathology scores in both the brain and spinal cord, compared to the mice receiving spleen MNCs alone (Fig. 2). This difference was significant in the brain ($p<0.05$, Mann–Whitney *U*). Demyelination was not observed (data not shown). Therefore, although clinical signs of disease were not readily apparent, the transfer of MNCs isolated from the brains of VV_{PLP}-primed CFA/*BP* challenged mice resulted in significant neuropathology, mainly in the brain, whereas the transfer of only MNCs isolated from the spleens of VV_{PLP}-primed CFA/*BP* challenged mice did not.

Cytokine production by brain and spleen MNCs

The ability of the brain MNCs to transfer CNS disease may rest on the types and levels of cytokines produced by these cells. The concentrations of IL-17A and IFN-γ produced and released into the cell culture supernatant by brain and spleen MNCs were assayed by ELISA. Spleen MNCs stimulated with Con A (positive control) were found to produce a large amount of IL-17A (1,478 pg/ml) in comparison to unstimulated spleen MNCs [no treatment (No Tx), 48 pg/ml] (Fig. 3a). Likewise, spleen MNCs stimulated with Con A were found to produce a large amount of IFN-γ (558 pg/ml) in comparison to unstimulated spleen MNCs (No Tx, 56 pg/ml) (Fig. 3b). Spleen MNCs expanded with CD3/CD28 beads produced a very small amount of IL-17A (5 pg/ml) and some IFN-γ (83 pg/ml), whereas brain MNCs expanded with CD3/CD28 beads produced a larger amount of IL-17A (209 pg/ml) and IFN-γ (425 pg/ml) (Fig. 3). Therefore, the brain MNCs that were able to transfer disease were found to also produce IL-17 and IFN-γ.

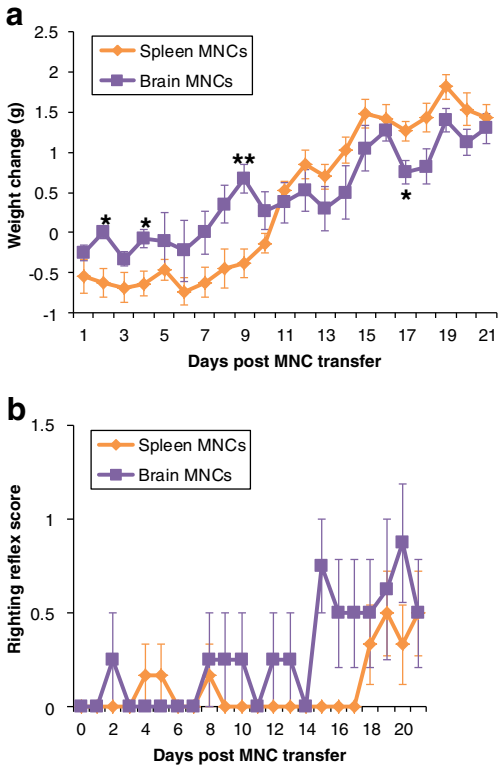

Fig. 1 Weight change and righting reflex as indicators of clinical disease following MNC transfer. MNCs, isolated from the brains and spleens of VV_{PLP}-primed CFA/*BP* challenged SJL/J mice, were expanded in vitro in the presence of CD3/CD28 beads and transferred into naïve SJL/J mice. Mice were monitored for clinical signs of disease. **a** Mice receiving brain MNCs had a significantly greater weight gain at days2, 4, and 9 post MNC transfer than mice receiving spleen MNCs alone. In contrast, mice receiving spleen MNCs alone had a significantly greater weight gain at day17 post MNC transfer than mice receiving brain MNCs. *$p<0.05$; **$p<0.01$, (ANOVA). Shown are mean weight changes from day0±standard errors of the means (SEM) for groups with four and six mice per group. **b** The mice receiving brain MNCs tended to have higher righting reflex scores than mice receiving spleen MNCs alone over the 3 weeks post MNC transfer; however, these differences did not reach significance. Shown are means±SEM for groups with four and six mice per group

Curdlan administration resulted in less severe clinical signs of disease

Here, we tested the ability of curdlan, an IL-17 inducer, to modulate the disease by replacing the CFA/*BP* challenge with either curdlan/IFA or curdlan/PBS. An initial examination of these mice showed that 100% of the curdlan/IFA-challenged mice and 40% of the curdlan/PBS-challenged mice had mild righting reflex disturbances (Libbey et al. 2010). However, when the weight changes (Fig. 4a) and righting reflex scores (Fig. 4b), both clinical signs of disease, for the curdlan/IFA and curdlan/PBS-challenged mice were compared on a daily basis to the CFA/*BP* challenged mice, with 5 to 19 mice per group, curdlan administration resulted in less severe disease. The VV_{PLP}-primed CFA/*BP*

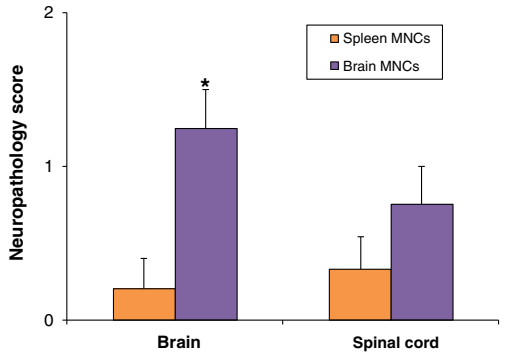

Fig. 2 Neuropathology following MNC transfer. MNCs, isolated from the brains and spleens of VV_{PLP}-primed CFA/*BP* challenged SJL/J mice, were expanded in vitro in the presence of CD3/CD28 beads and transferred into naïve SJL/J mice. Mice were euthanized at 3 weeks post MNC transfer and tissues were harvested, processed and stained with Luxol fast blue. Adjacent sections were immunostained for CD3. Neuropathology was scored as described in the "Materials and methods" section. The mice receiving brain MNCs had higher neuropathology scores in both the brain and spinal cord, compared to mice receiving spleen MNCs alone, and this difference was significant in the brain. *$p<0.05$ (Mann–Whitney *U*). Shown are means+SEM for groups with four and six mice per group

challenged mouse group had significantly greater weight loss on days 7 ($p<0.001$, ANOVA, Fisher's PLSD), 11 and 12 ($p<0.0001$, ANOVA, Fisher's PLSD), and 13 ($p<0.01$, ANOVA, Fisher's PLSD) post secondary challenge than both the VV_{PLP}-primed curdlan/IFA-challenged and the VV_{PLP}-primed curdlan/PBS-challenged mouse groups (Fig. 4a). On day 6 post secondary challenge, the VV_{PLP}-primed curdlan/PBS-challenged mouse group had significantly greater weight gain than both the VV_{PLP}-primed CFA/*BP* challenged and the VV_{PLP}-primed curdlan/IFA-challenged mouse groups (Fig. 4a, $p<0.01$, ANOVA). For righting reflex, the VV_{PLP}-primed CFA/*BP* challenged mouse group had significantly higher righting reflex scores than the VV_{PLP}-primed curdlan/IFA-challenged and the VV_{PLP}-primed curdlan/PBS-challenged mouse groups at days 12 and 13 post secondary challenge (Fig. 4b, $p<0.05$, Mann–Whitney *U*). Therefore, when CFA/*BP* secondary challenge of VV_{PLP}-primed SJL/J mice was replaced with

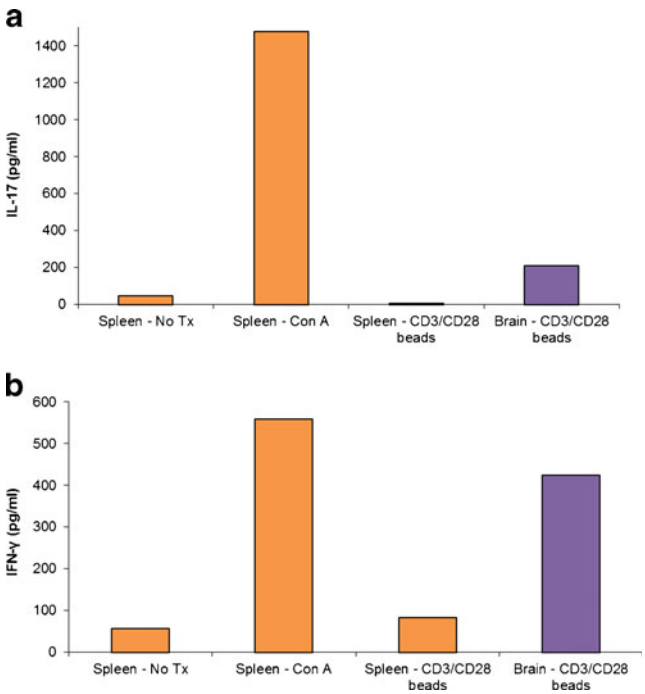

Fig. 3 Supernatant cytokine levels following CD3/CD28 bead stimulation of MNC cultures. MNCs, isolated from the brains and spleens of VV_{PLP}-primed CFA/*BP* challenged SJL/J mice, were expanded in vitro in the presence of CD3/CD28 beads. Supernatants were harvested and stored frozen until assayed. The concentrations of **a** IL-17A and **b** IFN-γ were measured in the cell culture supernatants by ELISA. No treatment (*No Tx*) and Con A stimulation of spleen MNCs served as negative and positive controls, respectively. The levels of both IL-17A and IFN-γ were higher in the brain MNCs expanded with CD3/CD28 beads than in the spleen MNCs expanded with CD3/CD28 beads

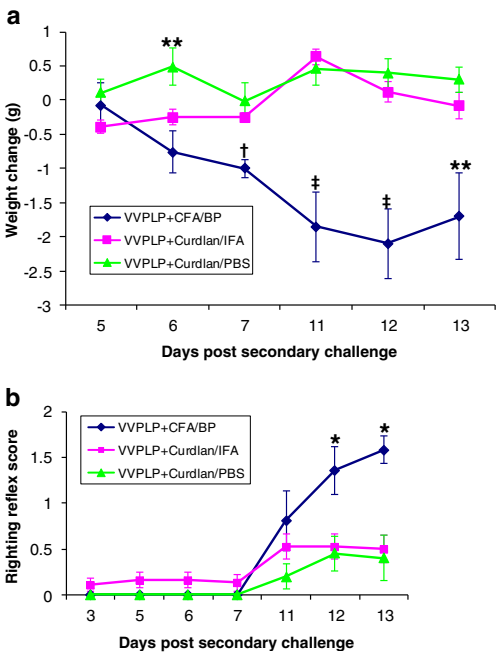

Fig. 4 Weight change and righting reflex as indicators of clinical disease following curdlan administration. Groups of VV_{PLP}-primed SJL/J mice were challenged, 5 weeks post infection, with CFA/*BP*, curdlan/IFA or curdlan/PBS and followed for clinical signs of disease. **a** At day 6 post secondary challenge, the VV_{PLP}-primed curdlan/PBS-challenged mouse group had significantly greater weight gain than both the VV_{PLP}-primed CFA/*BP* challenged and the VV_{PLP}-primed curdlan/IFA-challenged mouse groups. At days 7, 11, 12, and 13 post secondary challenge, the VV_{PLP}-primed CFA/*BP* challenged mouse group had significantly greater weight loss than the VV_{PLP}-primed curdlan/IFA-challenged and the VV_{PLP}-primed curdlan/PBS-challenged mouse groups. **$p<0.01$; †$p<0.001$; ‡$p<0.0001$ (ANOVA, Fisher's PLSD). Shown are mean weight changes from day 3±SEM for groups with 5 to 19 mice per group. **b** The VV_{PLP}-primed CFA/*BP* challenged mouse group had significantly higher righting reflex scores than the VV_{PLP}-primed curdlan/IFA-challenged and the VV_{PLP}-primed curdlan/PBS-challenged mouse groups at days 12 and 13 post secondary challenge. *$p<0.05$ (Mann–Whitney *U*). Shown are means±SEM for groups with 5 to 19 mice per group

curdlan/IFA or curdlan/PBS secondary challenge, the mice developed less severe disease.

Curdlan administration induced only mild neuropathology

Neuropathology was scored, as described in the methods, to determine whether neuropathology in the brains and spinal cords correlated with the clinical signs of disease. In general, the brains of all the groups (3 to 14 mice per group) scored higher for neuropathology than the spinal cords (Fig. 5). The VV_{PLP}-primed curdlan/PBS-challenged mouse group tended to have lower neuropathology scores in both the brains and spinal cords in comparison to the VV_{PLP}-primed CFA/*BP* challenged and the VV_{PLP}-primed curdlan/IFA-challenged mouse groups, although the differences between the groups did not reach significance (Fig. 5, $p>0.05$, Mann–Whitney *U*). At least in the case of the VV_{PLP}-primed curdlan/PBS-challenged mouse group, neuropathology was milder and this correlated with the clinical signs of disease. Demyelination was not observed (data not shown).

Curdlan administration induced IL-17 in the periphery

Curdlan has been shown to induce Th17 cells, which produce the signature cytokine, IL-17 (Zepp et al. 2011). For this reason, the levels of IL-17A in the sera were assayed by ELISA to confirm that curdlan administration resulted in an increase in the production of IL-17 in the periphery. The level of IL-17A in the sera of the VV_{PLP}-primed CFA/*BP* challenged mouse group was significantly lower than the level of IL-17A in the sera of both the VV_{PLP}-primed curdlan/IFA-challenged and the VV_{PLP}-primed curdlan/PBS-challenged mouse groups, with 9 to 16 mice per group (Fig. 6, $p<0.05$, ANOVA, Fisher's PLSD). Therefore, the administration of curdlan by two different routes (s.c. and i.p.) and in the presence of IFA or PBS resulted in an increase in the levels of IL-17 in the periphery.

Discussion

Previously, our group demonstrated that SJL/J mice subclinically primed with VV_{PLP} did not develop disease, but were susceptible to the development of a CNS inflammatory disease following secondary challenge with CFA/*BP* (Theil et al. 2001; Tsunoda et al. 2007). Further examination of this prime/challenge model of MS will expand our knowledge of the mechanism by which it may be possible for many different pathogens to be associated with MS. Towards this end, we examined the ability of MNCs, isolated from the brains and spleens of these VV_{PLP}-primed CFA/*BP* challenged mice, to both transfer disease and to produce IL-17 and IFN-γ, as a means of elucidating the encephalitogenic nature and phenotype of these MNCs. The MNCs isolated from the brains, but not the spleens, of these primed/challenged mice were found to both produce IL-17 and IFN-γ (Fig. 3) and to induce significant neuropathology within the brains of naïve SJL/J mice (Fig. 2), upon adoptive transfer,

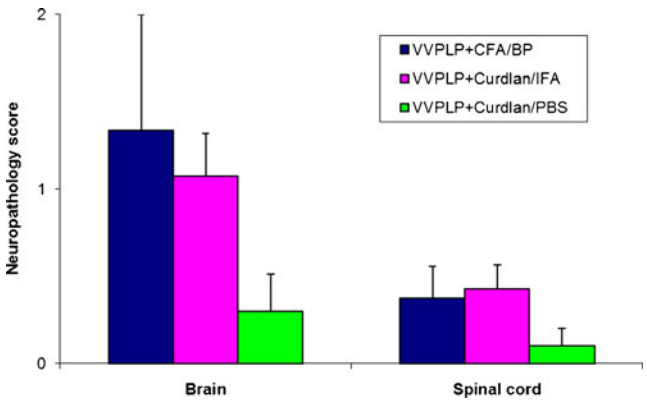

Fig. 5 Neuropathology following curdlan administration. Groups of VV_{PLP}-primed SJL/J mice were challenged, 5 weeks post infection, with CFA/*BP*, curdlan/IFA or curdlan/PBS. Mice were euthanized at 2 or 3 weeks post secondary challenge and tissues were harvested, processed and stained with Luxol fast blue. Adjacent sections were immunostained for CD3. Neuropathology was scored as described in the "Materials and methods" section. The VV_{PLP}-primed curdlan/PBS-challenged mouse group tended to have lower neuropathology scores in both the brain and spinal cord in comparison to the VV_{PLP}-primed CFA/*BP* challenged and the VV_{PLP}-primed curdlan/IFA-challenged mouse groups. Shown are means+SEM for groups with 3 to 14 mice per group

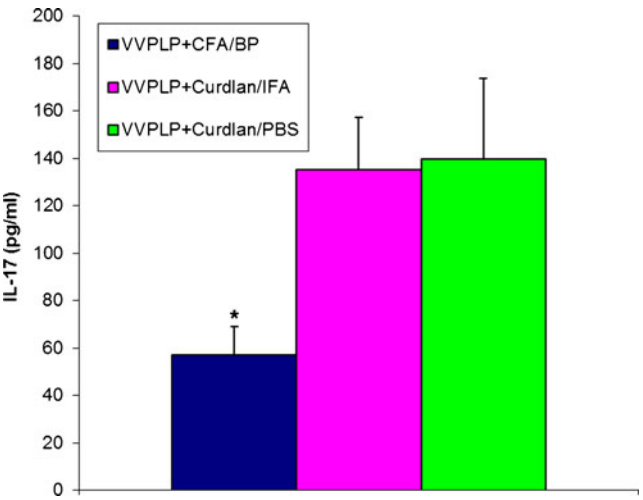

Fig. 6 Serum IL-17 levels following curdlan administration. Groups of VV_{PLP}-primed SJL/J mice were challenged, 5 weeks post infection, with CFA/*BP*, curdlan/IFA or curdlan/PBS. Mice were bled upon killing and the concentration of IL-17A was measured in the mouse sera by ELISA. The level of IL-17A in the sera of the VV_{PLP}-primed CFA/*BP* challenged mouse group was significantly lower than the level of IL-17A in the sera of both the VV_{PLP}-primed curdlan/IFA-challenged and the VV_{PLP}-primed curdlan/PBS-challenged mouse groups. *$p<0.05$, (ANOVA, Fisher's PLSD). Shown are means+SEM for groups with 9 to 16 mice per group

even in the absence of readily apparent clinical signs of disease (Fig. 1). Therefore, encephalitogenic cells were present within the brains of the VV_{PLP}-primed CFA/*BP* challenged mice and a subset of these cells were of the Th17 subset of $CD4^+$ T cells that has recently been implicated as effector cells in the EAE (Harrington et al. 2005) and TMEV models (Hou et al. 2009).

As examination of the MNCs suggested a role for IL-17, and therefore Th17 cells, in the development of the CNS inflammatory disease in VV_{PLP}-primed CFA/*BP* challenged mice, curdlan administration was employed as a means of further elucidating the encephalitogenic role of Th17 cells in this prime/challenge model. Curdlan induces Th17 cells [reviewed in (Laroche and Michaud 2007; McIntosh et al. 2005)], which have been implicated as effector cells in the EAE (Harrington et al. 2005) and TMEV models (Hou et al. 2009). Therefore, one would hypothesize that curdlan administration would exacerbate the CNS inflammatory disease; however, curdlan administration actually resulted in less severe disease, compared to CFA/*BP* injection. VV_{PLP}-primed mice were injected with curdlan, either s.c. in IFA or i.p. in PBS. Curdlan administration, via two different routes, in two different forms (IFA or PBS) and at two different concentrations, resulted in a reduction in clinical signs of disease (Fig. 4), and resulted in a reduction in neuropathology within the brains, at least in the VV_{PLP}-primed curdlan/PBS-challenged mice (Fig. 5). The VV_{PLP}-primed curdlan/PBS-challenged mice received 10 times more curdlan than the VV_{PLP}-primed curdlan/IFA-challenged mice (5 mg/mouse and 500 μg/mouse, respectively), which may explain the difference in the ability to reduce neuropathology in the brain between the two curdlan groups.

Curdlan was administered peripherally by two different routes: s.c. and i.p. In order to confirm the induction of Th17 cells in the periphery, IL-17A, the signature cytokine produced by Th17 cells, was assayed in the sera by ELISA. IL-17A, and therefore Th17 cells, were found to be induced in the periphery of VV_{PLP}-primed mice through both s.c. and i.p. administration of curdlan (Fig. 6). The induction of Th17 cells and IL-17A in the brains of VV_{PLP}-primed curdlan challenged mice was not determined.

The question remains: why did curdlan administration result in less severe disease instead of exacerbating disease as expected? The effect of curdlan administration may actually depend on many different factors. Some of these factors may include the timing and route of curdlan administration, the concentration of curdlan administered, the type of CNS inflammatory inducer used and the type of CNS inflammation induced. In this study of curdlan administration in VV_{PLP}-primed mice, the timing of curdlan administration was consistent at 5 weeks post VV_{PLP} infection, at which time the VV_{PLP} has been cleared. The route of administration was consistent in that both s.c. and i.p. are peripheral injections. As stated above, the VV_{PLP}-primed curdlan/PBS-challenged mice received ten times more curdlan than the VV_{PLP}-primed curdlan/IFA-challenged mice. Therefore, for the factors pertaining to curdlan, only the concentration of curdlan varied to any great extent. The inclusion of IFA as an adjuvant for the curdlan may also have played a role in disease modulation. Finally, in the case of the factors pertaining to the CNS inflammatory disease, the VV_{PLP}-prime CFA/*BP* challenge model may vary a great deal from the EAE and TMEV models. This is evidenced by the fact that VV_{PLP} infection did not induce disease, but did increase the susceptibility of the mice to the development of a CNS inflammatory disease following secondary challenge with CFA/*BP* (Theil et al. 2001; Tsunoda et al. 2007).

This examination of the effect of curdlan administration was not the first time that the reverse of what was expected was seen following an attempt to modulate the CNS inflammatory disease that develops in this prime/challenge model. Previously, VV_{PLP}-primed mice were injected with IL-12 at 5 weeks post VV_{PLP} infection (Tsunoda et al. 2007). IL-12 has been reported to enhance EAE (Gran et al. 2004), through its role in the generation of $CD4^+$ Th1 cells [reviewed in (Petermann and Korn 2011)], which were the effector $CD4^+$ T cells originally implicated in EAE. At 3 weeks post secondary challenge, none of the IL-12-injected mice developed clinical signs or substantial CNS pathology (Tsunoda et al. 2007).

Further examination of the VV_{PLP}-prime CFA/*BP* challenge model may suggest new, unexpected mechanisms for how many different viruses and microbes associated with MS can protect or enhance disease (Fujinami 2001). Towards this end, we are currently investigating: (1) whether CNS infiltrates are derived from transferred MNCs; (2) the phenotypes and cytokine profiles of CNS infiltrates in both MNC recipient mice and curdlan treated mice; and (3) the role of IL-17 through in vivo administration of blocking antibody against IL-17.

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