- Interleukin-6 control of early Theiler's Murine
- **2 Encephalomyelitis Virus replication in**
- macrophages occurs in conjunction with STAT1
- 4 activation and nitric oxide production.
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- Short Running Title:
- 24 Interleukin-6 and control of early TMEV infection

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Word Count: 5,615241 Words in Abstract

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47	Abbreviations:
48	IFN=interferon
49	IRF=interferon response factor
50	TMEV=Theiler's murine encephalomyelitis virus
51	MAPK=mitogen-activated protein kinase
52	ERK= extracellular signal-regulated kinase
53	EAE = experimental autoimmune encephalomyelitis
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ABSTRACT

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During Theiler's virus (TMEV) infection of macrophages it is thought that high IL-6 levels contribute to demyelinating disease found in chronically infected SJL/J mice but absent in B10.S mice capable of clearing the infection. Therefore, IL-6 expression was measured in TMEV-susceptible SJL/J and TMEV-resistant B10.S macrophages during their infection with TMEV DA strain or responses to LPS or poly I:C. Unexpectedly, IL-6 production was greater in B10.S macrophages than SJL/J macrophages during the first 24 h after stimulation with TMEV, LPS or poly I:C. Further experiments showed that in B10.S, SJL/J, and RAW264.7 macrophage cells, IL-6 expression was dependent on extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) and enhanced by exogenous IL-12. In SJL/J and RAW264.7 macrophages, exogenous IL-6 resulted in decreased TMEV replication, earlier activation of STAT1 and STAT3, production of nitric oxide, and earlier up-regulation of several anti-viral genes downstream of STAT1. However, neither inhibition of IL-6-induced nitric oxide nor knockdown of STAT1 diminished the early anti-viral effect of exogenous IL-6. In addition, neutralization of endogenous IL-6 from SJL/J macrophages with Fab antibodies did not exacerbate early TMEV infection. Therefore, endogenous IL-6 expression after TMEV infection is dependent on ERK MAPK, enhanced by IL-12, but too slow to decrease viral replication during early infection. In contrast, exogenous IL-6 enhances macrophage control of TMEV infection through preemptive anti-viral nitric oxide production and anti-viral STAT1 activation. These results indicate that immediateearly production of IL-6 could protect macrophages from TMEV infection.

INTRODUCTION

Interleukin (IL)-6 is a pleiotropic cytokine expressed by many cell types that is induced by microbes and other cytokines. IL-6 can play a beneficial role during immune response by contributing to neutrophil activity, nitric oxide production and development of the Th17 CD4 T cell subset, and a beneficial role in the brain (43) and neuronal health (9, 11). In contrast, persistent IL-6 induction during infection can contribute to detrimental effects on surrounding neurons or bone and poor control of cancer or autoimmune responses (24). During microbial induction of IL-6, activation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) MAPK signaling pathways contribute to IL-6 expression (21, 49) and stabilization of IL-6 mRNA (2) for protein translation.

Macrophages express IL-6 when they encounter microbes such as bacteria, fungi, or viruses (44). While the neutrophil- and Th17-promoting activity of IL-6 is critical in controlling certain bacterial or fungal infections, less is known about the role of IL-6 in viral infections. Recently, an antiviral role for IL-6 has been shown in mouse poxvirus infections, where early IL-6 production is required to control a potentially lethal infection (30), and in lymphocytic choriomeningitis virus infection, where late IL-6 production was shown to be critical for clearance of persistent viruses (12). However, failure to clear viruses from infected macrophages could result in viral spread to other tissues due to macrophage chemotaxis and could result in persistent IL-6 expression in affected tissue causing inflammatory pathologies and autoimmune diseases (16). Similarly, persistent elevation of IL-6 is associated with the development of experimental autoimmune

encephalomyelitis (EAE) in mice immunized peripherally with myelin peptides, whereas mice deficient in IL-6 are resistant to development of EAE (32, 40). Such data suggests that IL-6 expression may be essential to control early viral infection, yet contribute to pathology when expressed persistently.

Theiler's murine encephalomyelitis virus (TMEV) causes an acute infection that is cleared through innate and adaptive immune responses in most mouse strains yet produces debilitating sequelae in susceptible mice (33). C57Bl/6 and B10.S mice are prototypical TMEV-resistant strains whereas SJL/J mice fail to clear TMEV from macrophages and dendritic cells, resulting in persistent infection (25). Subsequent infection of macrophages in the central nervous system (CNS) of SJL/J mice leads to a demyelinating disease similar to human multiple sclerosis(5). Several reports have shown that a week after infection with TMEV, CNS macrophages, microglial cells, and astrocytes from SJL/J mice express IL-6 to a greater extent than C57Bl/6 mice (14, 17, 34). A proposed theory is that chronic production of IL-6 in the CNS of persistently infected SJL/J mice contributes to demyelinating disease in response to TMEV(17).

We propose that the dichotomy of TMEV persistence seen in resistant and susceptible macrophages depends on early differences in the cytokine production. We have previously shown that B10.S macrophages produce more IL-12 p70 in response to TMEV infection than SJL/J macrophages *in vitro* (37). Significantly, when SJL/J macrophages are pretreated with IL-12, TMEV replication is reduced to levels comparable with B10.S macrophages. Paradoxically, SJL/J macrophages express more interferon (IFN)β than B10.S macrophages before and during infection with

TMEV(37), yet this enhanced endogenous IFN β expression is insufficient to control TMEV replication. However, pretreatment with additional exogenous recombinant IFN β decreases TMEV replication in SJL/J macrophages to amounts seen in B10.S macrophages (37). We found that p38 ERK MAPK pathways are activated in response to TMEV infection of B10.S or SJL/J macrophages and play a role in IL-12 and IFN β expression (37). Therefore, differences in MAPKs, IL-12, and IFN β may be related to disparities in susceptibility of macrophages to persistent infection with TMEV.

In order to further understand the differences between SJL/J and B10.S mice that could account for differences in TMEV persistence, we examined IL-6 expression in macrophages during the early response to TMEV. Unexpectedly, we found that during early response to infection with TMEV, B10.S macrophages expressed significantly more IL-6 than SJL/J macrophages. Moreover, pre-treatment of macrophages with exogenous IL-6 reduced TMEV replication but neutralization of endogenous TMEV-induced IL-6 with Fab antibodies failed to affect TMEV replication in SJL/J macrophages. Additionally, we show that TMEV-induced IL-6 secretion in macrophages depends on ERK-MAPK and is enhanced by IL-12. However, neither IL-6-induced nitric oxide production nor STAT1 activation are essential to IL-6 reducing TMEV infection.

MATERIALS AND METHODS

Mice, virus, cell lines, and reagents. The eight to twelve week old female B10.S and SJL/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD) and

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maintained in DMEM with 10% FBS with 50 μg/ml gentamycin. The p38 MAP-kinase inhibitor SB203580 and ERK MAPK inhibitor U0126 were obtained from Promega Corporation (Madison, WI), E. coli LPS O127:B8 was obtained from Sigma Chemical Co.(St. Louis, MO), and poly I:C was obtained from InvivoGen (San Diego, CA). The inducible nitric oxide synthase inhibitor, L-NIL, was obtained from TOCRIS Bioscience (Bristol, UK) and incubated with macrophages where indicated at 10 μM. The nitric oxide donor spermine NONOate was obtained from Invitrogen (Carlsbad, CA) and incubated with macrophages where indicated at 1 mM. Affinity purified neutralizing rat antibody to mouse IL-6 (clone MP5-20F3) (48) was obtained from Invitrogen. Fab fragments of neutralizing anti-IL-6 were obtained after papain digestion using the Fab preparation kit of Pierce (Thermo Fisher Scientific, Rockford, IL). The initial stock of the DA strain of TMEV was obtained from Dr. Kristen Drescher, Department of Medical Microbiology and Immunology, Creighton University, Omaha, Nebraska. TMEV was grown in BHK-21 cells. The titer of stock cultures of TMEV was 2.5 x 10⁶ PFU/ml and macrophages cultures were infected with 2.5 x 10⁵ PFU of TMEV unless otherwise stated.

Macrophage preparations. Macrophages were elicited by intraperitoneal injection of 2 ml thioglycollate broth into mice. Four days later, the peritoneal cavities were each flushed with 2 ml DMEM and the cells were incubated at 1 x 10^6 cells/2 ml of DMEM cell culture medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen), and 50 μ g/ml gentamycin (Invitrogen). After 24 h, non-adherent cells were removed and 1 ml of culture medium added. Adherent cells were

greater than 90% Mac-1⁺ as determined by FACS analysis. These macrophages were either untreated or pretreated for 30 min with 20 μ M of SB203580, 40 μ M U0126, 1 ng/ml IL-12 p70 (p35/p40; BD-Pharmingen, San Diego, CA), 10 ng/ml IFN β (Minneapolis, MN) or 10 ng/ml IL-12 p40 (BD-Pharmingen). Untreated or pretreated macrophages were uninfected, infected with 100 μ l of the TMEV stock (2.5 x 10⁵ PFU), stimulated with 1 μ g/ml LPS, or stimulated with 50 μ g poly I:C or left unstimulated. After 3, 8, or 24 h of infection or stimulation, supernatants were collected for ELISA and cell extracts were collected for RNA preparation and quantitative RT-PCR.

RNA interference. Validated inhibitory siRNA targeting mouse STAT1 and STAT3 was purchased from Cell Signaling, Inc. (Danvers, MA) and transfected into RAW264.7 cells according to manufacturer's specifications using the nucleofection kit of Amaxa 36 h prior to challenge with TMEV.

RNA preparation and quantitative RT-PCR. RNA was extracted from cells using the RNAeasy kit of Qiagen (Valencia, CA), PerfectPure kit from 5Prime (Gaithersburg, MD), or the Purelink kit from Ambion/Invitrogen (Carlsbad, CA) according to the manufacturer's specifications. One-hundred ng to one μg of RNA was reverse transcribed in 0.5 mM each of dATP, dGTP, dTTP, and dCTP, 20 U of RNAse inhibitor with Superscript II reverse transcriptase (Invitrogen) at 42°C for 1.5 h followed by 94°C for 5 min. One twenty-fifth of the cDNA sample was incubated with 0. 4 μM of the following primer pairs (Invitrogen): IFNβ sense 5' ATGAACAACAG GTGGATCCTCC 3' and anti-sense 5' AGGAGCTCCTGACATTTCCGAA 3'; IL-6 sense 5' ATGAAGTTCCT CTCTGCAAGAGACT 3' and antisense 5' CACTAGGTTTGCC GAGTAGATCTC 3';

209	IRF1 sense 5' ATGCCAATCACTCGAATGCGGA 3' and antisense 5' GGCTGC
210	CACTCAGACTGTTCAA 3'; IRF-7 sense 5' CCAGCGAGTGC TGTTTGGAGAC 3' and
211	antisense 5' TTCCCTATTTTCCGTGGCTGGG 3'; IRF-9 sense 5' ATGGCCTCA
212	GGCAAAGTACGCT 3' and antisense 5' TTCGCTTGCATGG TGATTTCTG 3'; TMEV
213	sense 5' CTTCCCATTC TACTGCAATG 3' and antisense 5' GTGTTCCTGG
214	TTTACAGTAG3'; or GAPDH sense 5'-TTGTCAGCAA TGCATCCTGCAC-3' and
215	antisense 5'-ACAGCTTTCCA GAGGGGCCATC-3'. Quantitative PCR reactions were
216	run on an ABI Prism 7000 thermal cycler at 50 °C for 2 min, 95 °C for 10 min, 45 cycles
217	of 95 °C for 15 s/60 °C for 30 s. Cycle thresholds (Ct) of sample were normalized to Ct
218	of GAPDH for that sample (Δ Ct), and then normalized to the average Δ Ct of the control
219	samples ($\Delta\Delta Ct$) after which data are expressed as relative levels of mRNA using 2 ^{-$\Delta\Delta Ct$} .
220	When macrophages are pretreated, TMEV RNA data are reported as percentage of
221	TMEV RNA in non-treated but infected macrophages.
222	ELISAs. ELISA plates were coated with antibodies to mouse IL-6 (MP5-
223	20F3;BD-Pharmingen), the plates were blocked with PBS/10% FBS. After washes, cell
224	culture supernatants or serial dilutions of recombinant IL-6 (BD-Pharmingen) were
225	added to wells. After 2 h, biotinylated antibody to mouse IL-6 (MP5-32C11;BD-
226	Pharmingen) was added to each well. After 1 h, strepavidin horseradish peroxidase
227	(1:1000; BD-Pharmingen) was added for 30 min and then 3,3', 5, 5'
228	Tetramethylbenzindine substrate/hydrogen peroxide solution (BD-Pharmingen) was
229	added to each well. IL-6 was measured by determining optical densities at OD 450 nm
230	wavelength with reference OD 570 nm using an ELISA spectrophotometric plate reader

PAGE and western blot analysis. Cell lysates were obtained from RAW cells
challenged with TMEV with or without treatment with 10 ng/ml recombinant murine IL-6
(BD-Pharmingen, San Diego, CA) or murine recombinant IFN β (Interferon Source,
Piscataway, NJ). Twenty μl of each sample containing 20 μg of protein in sample buffer
with bromophenol blue was run on a 10% SDS, Tris-glycine- polyacrylamide gel and
transferred to a nitrocellulose membrane. The membrane was treated with blocking
buffer for 1 h at room temperature, followed by incubation in 1:500 dilution of rabbit IgG
anti-phospho-STAT1(Invitrogen, Camarillo, CA), 1:1000 dilution of anti-phospho-STAT3
(Cell Signalling, Beverly, MA), 1:500 dilution of anti-mouse STAT1(Invitrogen), 1:1000
dilution of anti-mouse STAT3 (Invitrogen), or 1:500 mouse anti-tubulin E7
(Developmental Studies Hybridoma Bank, University of Iowa) and then 1:5000 dilution
of IRDye® 800CWGoat Anti-Rabbit IgG (Rockland Immunochemicals, Inc.,Gilbertsville,
PA) or Alexa Fluor680-labeled anti-Mouse IgG (Rockland Immunochemicals). The
washed membrane was scanned with a LICOR Odyssey® Infrared Imaging System and
densitometric analysis done with LICOR imaging software.

Nitric Oxide Assay. Induction of nitric oxide (NO) was assayed in culture supernatants by measuring nitrite using the Greiss reagent kit of Invitrogen. Briefly, 20 μ L of Griess Reagent was mixed with 150 μ L of supernatant plus 130 μ L of deionized water and incubated for 30 min at room temperature. Color development at 570 nm, which is proportional to nitric oxide in supernatants, was measured with a spectrophotometer.

Statistical analysis. The Student's t test of the GraphPad Prism Software was used to determine the significance of differences between means; p < 0.05 was considered significant.

RESULTS

TMEV-induced IL- 6 production is greater in TMEV-resistant B10.S macrophages. IL-6 contributes to both antiviral immunity and virus-induced pathology (3, 41). TMEV is cleared from macrophages of TMEV-resistant mice but persists in (CNS) macrophages in susceptible SJL/J mice (5). Therefore, IL-6 mRNA and protein from TMEV-susceptible SJL/J and TMEV-resistant B10.S macrophages were determined following challenge with TMEV. IL-6 mRNA was detected at 3 and 8 h after TMEV challenge (Fig. 1 A) in both B10.S and SJL/J macrophages, however B10.S macrophages produced more IL-6 mRNA than SJL/J macrophages at 8 h after infection. Similarly, B10.S macrophages produced significantly more IL-6 protein 8 h after TMEV challenge compared with SJL/J macrophages in response to TMEV (Fig. 1 B). Because TMEV RNA replication is significantly higher in SJL/J macrophages compared with B10.S macrophages (37), the data here suggest that enhanced early IL-6 production may contribute to better control of TMEV replication.

To determine if exogenous IL-6 could impede TMEV replication in SJL/J macrophages during early infection, recombinant IL-6 was added to SJL/J macrophages 30 min prior to TMEV infection and either left in the media during infection or washed

out by changing the media just prior to infection. Treatment with 10 ng/ml exogenous IL-6 significantly reduced TMEV replication at 8h post infection (PI) in SJL/J macrophages (Fig. 1C). In addition, treatment with as little as 0.1 ng/ml of exogenous IL-6 significantly reduced TMEV RNA at 24 h PI in SJL/J macrophages (Fig. 1D). Equally significant, IL-6 preemptively triggered anti-viral activity in SJL macrophages even when exogenous IL-6 was added for only 30 min and washed out prior to infection (Fig. 1D). These findings suggest that early differences in the amount of IL-6 present during the course of infection play an important role in the establishment of TMEV in SJL/J macrophages.

To examine whether TMEV-induced endogenous IL-6 contributes to control of TMEV replication in SJL/J mice, neutralizing anti-IL-6 (IgG; clone MP5-20F3) antibody was added to SJL/J macrophages at the time of TMEV infection (48). Surprisingly, addition of either whole anti-IL-6 antibody or isotype control antibody both resulted in a significant reduction in TMEV replication (see Fig. S1A and S1B). These findings do not preclude an antiviral role for IL-6, since interactions between the IgG antibody and Fcγ receptors may also induce anti-viral immunity in macrophages. Indeed addition of isotype antibody had a similar effect on TMEV infection of macrophages. Therefore we treated the whole antibody with papain and removed the Fc fragments to generate Fab anti-IL-6. Addition of Fab anti-IL-6 to SJL/J macrophages at the time of infection did not decrease but also did not increase TMEV replication in infected SJL/J macrophages (Fig. 1E).

To determine the time frame at addition in which exogenous IL-6 is most effective at reducing TMEV RNA we added 10 ng/ml recombinant IL-6 at 1, 3, 6, and 7 h after or exogenous IFNβ 7h PI of SJL/J macrophages with TMEV. Addition of exogenous IL-6 at 1 and 3 h PI significantly reduced TMEV RNA compared with untreated SJL/J macrophages that were infected with TMEV (Fig. 1F). Addition of IL-6 at 6 and 7 h PI failed to significantly reduce TMEV RNA in SJL/J macrophages compared to untreated macrophages. In contrast addition of IFNβ at 7 h PI significantly reduced TMEV RNA in SJL/J macrophages. Thus, while small amounts of added IL-6 were capable of controlling TMEV replication in SJL/J macrophages in vitro, it appears that a sufficient quantity of endogenous IL-6 is not produced quickly enough to provide protection because the beneficial effects of IL-6 are only evident prior to 6 h PI. These results confirm that the antiviral effect of IL-6 is most effective very early during the course of TMEV infection of macrophages. These findings warrant further investigation into strain differences in early TMEV-induced IL-6 expression.

TMEV-induced IL-6 expression in macrophages is dependent on ERK

MAPK. The cell signaling pathway activated by TMEV infection leading to IL-6

expression in macrophages is not well understood. We previously showed that TMEV

infection of macrophages from both B10.S and SJL/J mice strongly activates ERK

MAPK and weakly activates p38 MAPK (37). Therefore, macrophages from B10.S and

SJL/J mice were pretreated with ERK and p38 MAPK inhibitors 30 min prior to TMEV

infection. Twenty-four h after infection, the amount of TMEV-induced IL-6 protein

secreted by B10.S macrophages was significantly greater than that secreted by SJL/J

macrophages (Fig. 2 B). In contrast the 24 h TMEV-induced IL-6 mRNA was significantly higher in SJL/J macrophages than B10.S macrophages, suggesting translational control of IL-6. Moreover, TMEV-induced IL-6 mRNA expression was increased in macrophages treated with the p38 MAPK inhibitor, SB203580, (Fig. 2 A) but IL-6 protein production was unchanged (Fig. 2 B). Pretreatment of macrophages with the ERK MAPK inhibitor, U0126, significantly decreased TMEV induction of IL-6 mRNA (Fig. 2 A) and protein (Fig. 2 B) in both SJL/J and B10.S macrophages responding to TMEV. These results indicate that ERK MAPK activation is required for IL-6 expression in response to TMEV infection and suggest that TMEV-induced IL-6 expression is under translational control.

We have also shown that the TLR3 pathway is involved in macrophage cytokine responses to TMEV infection by inducing IFNβ, IL-12, and IL-23 (1). Therefore, we compared the response of macrophages from each strain when treated with the TLR3 agonist, polyI:C or the TLR4 agonist, LPS. B10.S macrophages expressed significantly more IL-6 mRNA and protein than SJL/J macrophages treated with LPS (Fig. 3 A, B) or polyI:C (Fig. 3 C, D), but in macrophages from both strains the level of IL-6 expression in response to poly I:C was substantially less than that in response to LPS or TMEV. Because ERK MAPK is activated by both TLR3 agonists (see Fig. S2) and TLR4 agonists (37), macrophages were also pretreated with U0126. Inhibition of ERK MAPK activation with U0126 significantly reduced IL-6 expression to the same extent in both B10.S and SJL/J macrophages responding to LPS and significantly decreased IL-6 production from SJL/J macrophages responding to polyI:C. Therefore, ERK MAPK

activation is required for IL-6 expression that occurs from activation of TLR3 and TLR4 pathways in SJL/J macrophages.

12. Previously, we showed that SJL/J macrophages responding to TMEV express significantly more IFNβ and IL-12 p40 (p40/p40) but significantly less IL-12 p70 (p35/p40) than B10.S macrophages. In addition we showed that addition of IFNβ, IL-12 p40, or p70 decreases TMEV replication in SJL/J macrophages (37). To see if these cytokines could affect TMEV-induced IL-6, B10.S and SJL/J macrophages were pretreated with the IL-12 p40 homodimer, bioactive IL-12 p70, or IFNβ 30 min prior to and during TMEV challenge. Treatment with IL-12 p70 or p40 significantly enhanced IL-6 expression in response to TMEV in both B10.S and SJL/J macrophages at 24 and/or 48 h post TMEV infection (Fig. 4 A, B), however the modest increase in IL-6 expression upon pretreatment with IFNβ was not significant (Fig. 4). As we have seen before, IL-12 and IFNβ treatment reduced TMEV replication in both B10.S and SJL/J macrophages (Fig. 4C). Therefore deficient production of IL-12 during response to TMEV could contribute to diminished IL-6 expression by SJL/J macrophages.

To gain further insight into the role of IL-12 in IL-6 expression, we used the RAW264.7 macrophage cell line which is permissive for TMEV replication, expresses IL-6 well, expresses IL-12 poorly, and activates ERK MAPK following TMEV infection (26, 37). To confirm the effects of IL-12 and the ERK MAPK inhibitor on IL-6 expression, RAW264.7 cells were treated with IL-12 with or without U0126 during TMEV infection. Pretreatment with IL-12 enhanced IL-6 mRNA (Fig. 5 A) and protein (Fig. 5 B)

expression following TMEV challenge of RAW264.7 cells, while pretreatment with
U0126 repressed the IL-12 enhancement of IL-6 (Fig. 5 A, B). Therefore IL-12 is
involved in IL-6 expression during TMEV infection of macrophages in an ERK MAPK
dependent manner.

IL-6 has direct antiviral activity against TMEV replication in RAW264.7 cells. We next set out to determine if, when, and at what quantity IL-6 represses TMEV infection in RAW264.7 cells. First, RAW264.7 cells were treated with 10 ng/ml exogenous recombinant IL-6 at 30 min prior to and during challenge with TMEV. Like SJL/J macrophages, treatment of RAW264.7 cells with IL-6 significantly decreased TMEV RNA at 24 h PI (Fig. 6 A). Interestingly, as little as 0.1 ng/ml significantly reduced TMEV RNA 24 h PI, (Fig. 6B). Furthermore, IL-6 treatment enhanced IL-6 expression (Fig. 6C) at 24 h PI, but IL-6 treatment did not decrease TMEV RNA at 3 and 8 h PI in RAW264.7 cells (Fig. 6D), suggesting the time period in which TMEV induced events occur in RAW264.7 are slightly different than that seen in SJL/J macrophages.

IL-6 antiviral activity against TMEV replication is associated with activation of STAT1 related anti-viral activity. Like IFN β , treatment of macrophages with IL-6 leads to activation of STAT3 by phosphorylation at its tyrosine 705 and activation of STAT1 at its tyrosine 701 (13). IL-6 activation of STAT1 could contribute to control of virus replication (28, 46) by inducing expression of IRF1(22), IRF7(23), and IRF9 that enhance IFN β expression. To determine if IL-6 activates STAT1 and induces expression of IRFs that are downstream of STAT1 during TMEV infection of

macropnages, RAW264.7 cells were treated with IL-6, IFNB, or TMEV alone or in
combination and phospho-STAT1, as well as phoshpo-STAT3 were evaluated by
western blot. TMEV infection alone failed to activate STAT1 by 30 min PI but did so by
6 h PI (Fig. 7A). In contrast, treatment of RAW264.7 cells with either IL-6 or IFN $\!\beta$
activated STAT1 and STAT3 as early as 30 min after TMEV challenge. To determine if
STAT1 activation is involved in control of TMEV replication, RAW264.7 cells were
transfected with small inhibitory RNA that has been verified to reduce STAT1 (siSTAT1)
or siSTAT3. Knockdown of STAT1 resulted in significantly increased TMEV RNA while
knockdown of STAT3 failed to affect TMEV RNA replication in RAW264.7 cells (Fig.
7B). However, pretreatment of STAT1-knocked-down RAW264.7 cells with exogenous
IL-6 resulted in a reduction of TMEV infection back to a level seen in RAW264.7 cells
infected with TMEV. Moreover, addition of exogenous IL-6 to STAT3-knocked down
and STAT1/STAT3 double-knocked down RAW264.7 cells significantly reduced TMEV
RNA replication compared with untreated RAW264.7 cells infected with TMEV (Fig. 7B)
Therefore, STAT1 activation contributes to control of TMEV replication and the
activation of STAT3 may negatively impact the antiviral effect of STAT1. Consistent
with STAT1 activation, treatment of RAW264.7 cells with IL-6 resulted in significant
enhancements of TMEV-induced IRF1 (Fig. 7C), IRF7 (Fig. 7D), IRF9 (Fig. 7E), and
IFN β (Fig. 7F). These results confirm that IL-6 can directly limit viral replication in
macrophages early after infection with TMEV by initiating a STAT1 anti-viral program.

a potent anti-viral factor (41). Therefore, another possibility is that IL-6 controls TMEV replication in macrophages by inducing nitric oxide. To explore this possibility, TMEV-challenged RAW264.7 cells were treated with 10 ng/ml IL-6 in the presence or absence of L-NIL, an inducible nitric oxide synthase inhibitor (27), and production of nitric oxide was measured. TMEV infection of RAW264.7 cells did not result in significant nitric oxide production at 24 h PI (see Fig. S3A). However, addition of exogenous IL-6 to TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric oxide secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide donor, repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L-NIL did not reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, while IL-6 induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive role in the IL-6-induced antiviral effect observed in macrophages in vitro.

DISCUSSION

The data herein show that IL-6 protects macrophages early after infection with TMEV by decreasing virus replication in macrophages. These results are somewhat surprising because research on chronic TMEV infection has supported the theory that enhanced IL-6 may play a detrimental role in the immunopathology of TMEV in susceptible mice (14). Indeed, a recent report has shown that SJL/J mice infected with TMEV exhibit greater levels of IL-6 in the CNS than TMEV-resistant C57BI/6 mice starting at day 8 after infection (14). However, in that report IL-6 was not measured until day-8 after infection. We show herein that the antiviral effect of IL-6 takes place within the first 6 h

after infection. Therefore the beneficial effect of IL-6 is early after TMEV infection, but
not later. It is also possible that TMEV-resistant C57Bl/6 mice may have a different
mechanism to control TMEV infection in macrophages compared with TMEV-resistant
B10.S mice. The results herein indicate that during the first day after infection in vitro,
IL-6 protein production by macrophages from TMEV-resistant B10.S mice is greater
than from macrophages of TMEV-susceptible SJL/J and this difference may play an
important role in determining the severity of the infection. Moreover, significant levels of
IL-6 are not produced until 8 h after TMEV infection and IL-6 is only effective at
controlling TMEV replication during the first 8 h after infection. Therefore, IL-6 produced
by the TMEV infected macrophages helps to protect uninfected macrophages from
subsequent infection. Altogether, the present data suggest the CNS demyelination that
SJL/J mice develop several weeks to months after infection with TMEV may be due in
part to an inability of SJL/J macrophages to control viral replication very early after the
initial infection. Similarly, B10.S mice that do not develop demyelination following
infection with TMEV may stem from better early control of TMEV replication in
macrophages. Our results indicate that ability to control TMEV replication in
macrophages begins during the first 24h after infection is to a large extent dependent on
macrophage production of IL-6, which then protects uninfected cells from infection.
Thus, IL-6 production by virally infected cells may have dual roles in viral infection by
contributing to both anti-viral immunity and subsequent pathology.

Several reports have proposed beneficial effects of exogenous IL-6 treatment in TMEV infection of SJL/J mice (35, 39). In fact, recombinant exogenous IL-6 was shown to suppress chronic demyelination and reduce virus replication in SJL/J mice infected

with TMEV. Other reports have shown that IL-6 is involved in neuronal health facilitating neuronal differentiation, neurite outgrowth, survival, regeneration (47) and oligodendrocyte differentiation (52). In stark contrast to these reports are others which have suggested that chronic expression of IL-6 in TMEV-susceptible SJL/J mice is responsible for development of demyelinating disease (17). Hou et al. (14) showed that IL-6 levels in the brain and spinal cord of SJL/J mice are elevated 8-90 days post infection compared with TMEV resistant C57Bl/6 mice. It is possible that the lower early acute IL-6 response of SJL/J mice to TMEV infection is inadequate to control viral replication, thereby rendering these mice susceptible to chronic viral infection and a chronically elevated IL-6 response. Alternatively, enhanced IL-6 in the CNS of TMEV infected SJL/J mice may not be a reflection of a heightened IL-6 response of infected cells to TMEV but may be due to enhanced infiltration of macrophages into the CNS, which then are infected with TMEV and produce IL-6.

The anti-viral and pathological effects of IL-6 notwithstanding, the signaling pathways that lead to IL-6 expression in response to TMEV remain unclear. Previous reports showed that ERK and p38 MAPK pathways are involved in both innate anti-viral immunity (7) and IL-6 expression (49). The results herein confirm that activation of ERK MAPKs contributes to IL-6 expression in response to TMEV. However, maximum ERK activation occurs within 30 min after TMEV infection of macrophages and we have not noticed differences in the intensity of ERK activation between SJL and B10.S macrophages following TMEV infection (37, 38). Activated ERK MAPKs have been shown to phosphorylate downstream transcription factors such as the cyclic AMP response element binding protein (CREB) at serine 133 (4, 51, 45), CREB response

elements (CRE) are located at the IL-6 promoter (8), activated CREB binds to IL-6 promoter CRE (10), and CREB activity is required for IL-6 (36). Therefore, TMEV induction of IL-6 is likely to depend on the ERK activation of the transcription factor, CREB.

The results herein also suggest that TMEV induction of IL-6 in macrophages is in part dependent upon macrophage production of IL-12, which is also the primary inducer of Th1 development during adaptive immune responses (15). In addition, our results show that IL-12 enhancement of IL-6 expression is dependent on the ERK-MAPK pathway. Other reports have shown in IL-12 p35 knockout mice that IL-12 plays a significant role in IL-6 expression during viral infection (18), IL-6 expression depends in part on activation of ERK (19), and that IL-12 activates ERK-MAPK (20). These results are consistent with our previous data that showed significantly more IL-12 production from B10.S macrophages compared with SJL/J macrophages following TMEV infection (37). Therefore, IL-12 expression by macrophages that is responsible for Th1 development during adaptive immune responses also contributes to IL-6 expression during innate immune responses, both roles of IL-12 are likely to be beneficial to early anti-viral immunity.

Overall the data here suggest a direct, interferon-like role for IL-6 in the suppression of TMEV replication, which is exemplified by its ability to activate STAT1 and induce expression of STAT1 dependent genes. Indeed early reports regarding IL-6, which was originally called Interferon-beta 2, showed it to have anti-viral activity (44). While it is not clear how IL-6 decreases TMEV replication, it is known to be a potent

inducer of iNOS and nitric oxide production (31), which has anti-viral properties (41). Our data confirm that IL-6 induces significant nitric oxide production from macrophages and that nitric oxide does indeed control TMEV replication. However, preventing IL-6-induced nitric oxide production did not reverse the anti-viral effect of IL-6. This is most likely because IL-6 also activated STAT1 and STAT3 that lead to the induction of STAT1-downstream anti-viral genes, IRF1, IRF7, and IRF9. Therefore the data suggest that early after infection with TMEV, IL-6 controls acute viral infection by inducing multiple innate antiviral programs.

One of the discrepancies noted herein is that between IL-6 mRNA expression and IL-6 protein secretion at time points beyond 8 h post infection. These data suggest that TMEV induced IL-6 production is under translational or inhibitory RNA control, which is especially notable in the TMEV-susceptible SJL/J macrophages. At 24 and 28 h post infection SJL/J macrophages expressed more IL-6 mRNA but produced less IL-6 compared with B10.S macrophages. In one study K homology (KH)-type splicing regulatory protein (KHSRP) was shown to target the AU-rich elements in the IL-6 mRNA 3' untranslated region which restricts its translation but does not lead to degradation of mRNA (6). In another study microRNA-365 was shown to inhibit the translation of IL-6 mRNA without affecting IL-6 mRNA (50). Our results suggest that differences in translational or inhibitory control of IL-6 expression following infection of macrophages could contribute to susceptibility versus resistance of macrophages to TMEV.

In summary, our findings show that strain differences in onset and amount of IL-6 upregulation upon infection with TMEV correlate with previously observed differences in

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disease outcomes between B10.S and SJL/J mice. Whereas B10.S mice with enhanced early IL-6 are capable of clearing TMEV, in SJL/J mice TMEV replication is not reduced by IL-6 unless exogenous IL-6 is added, suggesting that these mice do not produce IL-6 quickly enough or in sufficient quantity to control viral replication. The present investigation suggests that enhanced early expression of IL-6 by macrophages during infection with a macrophage-trophic virus, TMEV, is responsible for better control of viral replication in TMEV-resistant B10.S macrophages compared with TMEVsusceptible SJL/J macrophages. These results indicate that the persistent TMEV infection of macrophages in the CNS of SJL/J mice could be the result of insufficient IL-6 production that controls early TMEV infection through multiple anti-viral mechanisms that include nitric oxide production and STAT1 activation. Furthermore, the present data clearly shows that IL-12 and ERK MAPK play a major role in TMEV-induced IL-6. While ERK MAPK is required for IL-6 expression in response to TMEV its activation is equivalent in both strains. In contrast our previous studies point to differences in TLR signaling and IL-12 expression that may contribute to strain differences in TMEVinduced IL-6. These results are supported by our data showing that TMEV-induced IL-6 is also antiviral in RAW264.7 cells, IL-6 expression is enhanced by IL-12, and IL-6 leads to activation of STAT1, expression of its downstream genes IRF1, IRF7, IRF 9, and IFNbeta, as well as induction of nitric oxide. These results are significant because persistent infection of SJL/J macrophages by TMEV contributes to the inflammatory autoimmune demyelination in the CNS of TMEV infected SJL/J mice, which mimics human Multiple Sclerosis, that is not seen in TMEV infected B10.S mice. It remains to be determined if IL-6 is effective at controlling TMEV replication during established

539 persistent TMEV infection of macrophage populations. Nevertheless, this study suggests therapeutic strategies that promote early IL-6 antiviral pathways could prevent 540 chronic viral infections. 541 542 **ACKNOWLEDGEMENTS** 543 This work was supported by funding from the University of Nebraska Medical Center 544 College of Dentistry and University of Nebraska Lincoln, School of Biological Sciences, 545 and supported by Award Number 8P30GM10350903 and 5P20GM103489 from the 546 547 National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). The content is solely the responsibility of the authors and 548 does not necessarily represent the official views of the National Center for Research 549 Resources or the National Institutes of Health. 550 551 **REFERENCES** 552 553 Al-Salleeh, F., and T. M. Petro. 2007. TLR3 and TLR7 are involved in 1. 554 expression of IL-23 subunits while TLR3 but not TLR7 is involved in expression 555 of IFN-beta by Theiler's virus-infected RAW264.7 cells. Microbes Infect 9:1384-556 557 92. 558 2. Andoh, A., M. Shimada, S. Bamba, T. Okuno, Y. Araki, Y. Fujiyama, and T. Bamba. 2002. Extracellular signal-regulated kinases 1 and 2 participate in 559

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739	FIG 1 TMEV induces more IL-6 expression in macrophages. IL-6 mRNA or protein
740	secretion was induced from SJL/J or B10.S macrophages 3 and 8 h after infection with
741	TMEV. SJL/J or B10.S macrophages (1 x 10^6) were infected with 2-10 x 10^5 PFU of
742	TMEV in the absence (A, B), or presence of 10 ng/ml recombinant (rec) IL-6 (C, E),
743	0.01–10 ng/ml rec IL-6 (D), 3 μ g/ml anti-IL-6-Fab (E), or SJL/J macrophages were
744	treated with 10 ng/ml rec IL-6 which was removed after 30 min (D), or SJLJ
745	macrophages were treated with 10 ng/ml rec IL-6 or rec IFN β starting at 1, 3, 6, or 7 h
746	PI (F). RNA was reverse transcribed and relative levels of IL-6 mRNA (A) was evaluated
747	by real-time PCR, IL-6 protein by ELISA (B), and TMEV by real-time PCR (C, D, E,F).
748	Data are means of 3-5 samples per time point evaluated by the Student t test; *
749	indicates $p \le 0.05$, ** $p \le 0.01$.
750	
751	FIG 2 ERK MAPK activation is required for optimum TMEV-induced IL-6 expression. 1
752	x 10^6 macrophages were untreated (nil or control) or pretreated with 20 μM SB203580
753	(SB) or 40 μM U0126 (U) for 30 min before infection with 2 x 10^5 PFU of TMEV. After 24
754	h, relative levels of IL-6 mRNA was evaluated by real-time PCR (A), IL-6 protein
755	secretion evaluated by ELISA (B), and TMEV RNA by real-time PCR (C). Data are
756	means of 5 samples from two experiments evaluated by the Student t test. * with
757	brackets indicates $p \le 0.05$.

760	FIG 3 Optimum IL-6 expression in B10.S compared with SJL/J macrophages following
761	TLR4 and TLR3 stimulation depends on ERK MAPK activation. 1 x 10 ⁶ macrophages
762	were untreated (control) or pretreated with U0126 (U) for 30 min before stimulation with
763	1 μ g/ml LPS (A, B) or 50 μ g/ml polyl:C (C, D). After 24 h (A, C), relative levels of IL-6
764	mRNA was evaluated by real-time PCR and (B, D) IL-6 protein secretion evaluated by
765	ELISA. Data are means of 5 samples evaluated by the Student <i>t</i> test. * indicates
766	comparisons considered significantly different; $p \leq 0.05$.
767	
768	FIG 4 IL-12 and IFN β enhance IL-6 expression and decrease TMEV replication during
769	TMEV infection of B10.S and SJL/J macrophages. 1 x 10 ⁶ macrophages from SJL/J and
770	B10.S mice were untreated (control) or treated with IL-12 p70 (1 ng/ml), IFN β (10
771	ng/ml), or IL-12 p40 (10 ng/ml) 45 min before and during infection with 2 x 10^5 PFU of
772	TMEV. After 24 and 48 h of infection relative levels of IL-6 mRNA was evaluated by
773	real-time PCR (A), IL-6 protein was evaluated by ELISA (B), and TMEV RNA was
774	evaluated by real-time PCR (C). Data are means of 3 samples each evaluated by the
775	Student t test. * indicates comparisons considered significantly different; $p \le 0.05$.
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780	FIG 5 IL-12 and ERK-MAPK contribute to IL-6 expression during TMEV infection of
781	RAW264.7 macrophages. 1 x 10 ⁶ RAW264.7 macrophage cells were untreated (control)
782	or treated with IL-12 p70 (1 ng/ml) with or without 40 μM U0126 ERK MAPK inhibitor 45
783	min before and during infection with 1 \times 10 6 PFU of TMEV. After 24 h of infection
784	relative levels of IL-6 mRNA (A) was evaluated by real-time PCR and (B) IL-6 protein
785	was evaluated by ELISA. Data are means of 3 samples each evaluated by the Student t
786	test. This experiment was repeated three times. * indicates $p \le 0.05$, ** $p \le 0.01$, n.d.=
787	not detectable.
788	
789	FIG 6 IL-6 constrains TMEV replication during infection of RAW264.7 macrophages. 1
790	x 10 ⁶ RAW264.7 macrophage cells were untreated (control) or treated with 10ng/ml IL-6
791	(A, C) or 0.01- 10 ng/ml IL-6 (B) during infection with 1 x 10^6 PFU of TMEV. After 3, 6
792	(D), or 24 h (A, B, C) of infection relative levels of TMEV (A, B, D) or IL-6 (C) mRNA
793	were evaluated by real-time PCR. Means are of 6 samples from two experiments each
794	evaluated by the Student t test. * indicates $p \le 0.05$, ** $p \le 0.01$.
795	
796	FIG 7 IL-6 activates STAT1 and STAT3 in macrophages. (A) Western blots of
797	phospho-STAT1, phospho-STAT3, total STAT3, and beta-tubulin at 30 min and 6 h in
798	untreated or RAW264.7 cells treated with IL-6 or IFN β with or without TMEV infection.
799	(B) TMEV RNA measured by qRT-PCR in RAW264.7 cells transfected with siSTAT1-

RNA (3 pmol) or siSTAT3-RNA (3 pmol) and then infected with TMEV at 1 MOI after 36

801	h. Real-time PCR of IRF1 (B), IRF7 (C), IRF9 (D), and IFNβ (E) in RAW cells treated
802	with 10 ng/ml IL-6 prior to and during TMEV infection after 3 and 6 h infection. Data are
803	means of 5 samples each evaluated by the Student <i>t</i> test. * indicates means
804	considered significantly different; $p \le 0.05$; n.d.= not detectable.
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Figure 1. Moore et al.

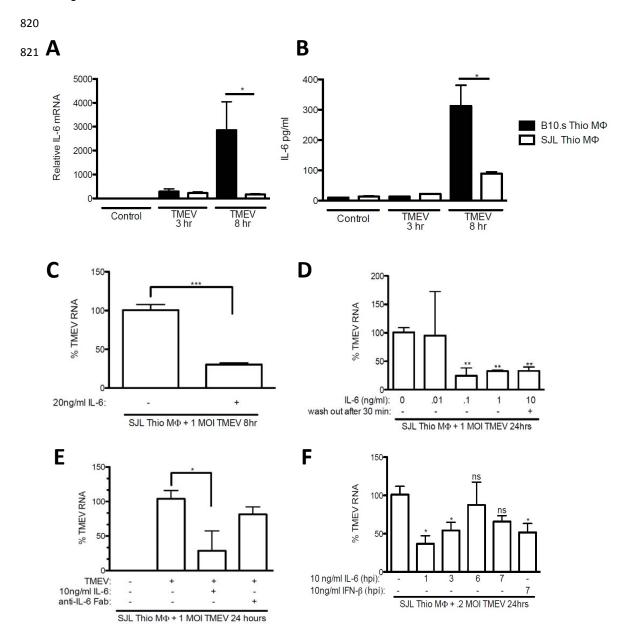


Figure 2. Moore et al.

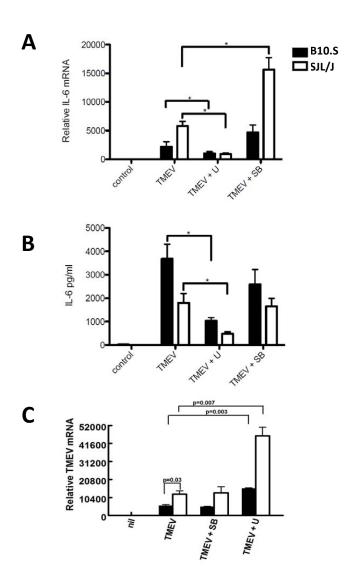


Figure 3. Moore et al.

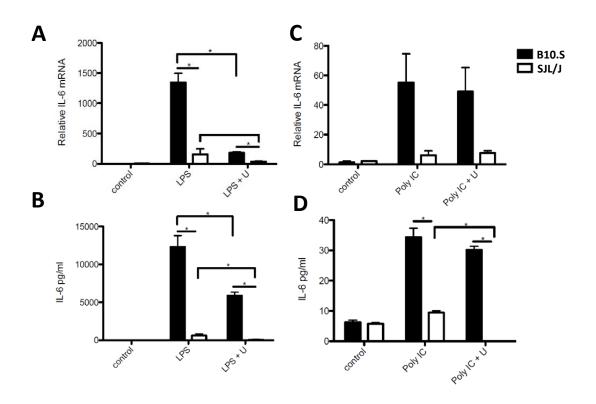
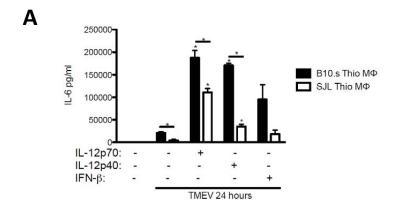
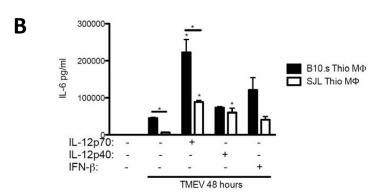


Figure 4. Moore et al.





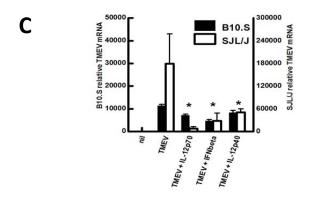


Figure 5. Moore et al.

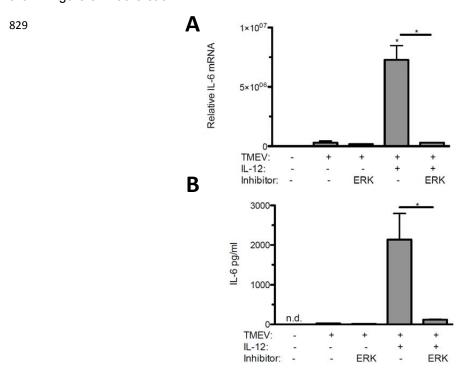


Figure 6. Moore et al.



