University of Nebraska - Lincoln DigitalCommons@University of Nebraska - Lincoln

Virology Papers

Virology, Nebraska Center for

2012

Interleukin-6 control of early Theiler's Murine Encephalomyelitis Virus replication in macrophages occurs in conjunction with STAT1 activation and nitric oxide production.

Tyler C. Moore University of Nebraska-Lincoln, tylermooreunl@huskers.unl.edu

Katherine L. Bush University of Nebraska Medical Center

Deborah Brown University of Nebraska - Lincoln, dbrown7@unl.edu

Thomas M. Petro University of Nebraska Medical Center, tpetro@unmc.edu

Follow this and additional works at: http://digitalcommons.unl.edu/virologypub Part of the <u>Virology Commons</u>

Moore, Tyler C.; Bush, Katherine L.; Brown, Deborah; and Petro, Thomas M., "Interleukin-6 control of early Theiler's Murine Encephalomyelitis Virus replication in macrophages occurs in conjunction with STAT1 activation and nitric oxide production." (2012). *Virology Papers*. Paper 228. http://digitalcommons.unl.edu/virologypub/228

This Article is brought to you for free and open access by the Virology, Nebraska Center for at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Virology Papers by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Interleukin-6 control of early Theiler's Murine 1

- **Encephalomyelitis Virus replication in** 2
- macrophages occurs in conjunction with STAT1 3
- activation and nitric oxide production. 4
- 5 6
- Tyler C. Moore^a, Katherine L. Bush^c, Liz Cody^{a,b}, Deborah M. Brown^{a,b}, and 7
- Thomas M. Petro^{b,c,#}. 8
- ^aSchool of Biological Sciences and [†]Nebraska Center for Virology, 9
- University of Nebraska Lincoln 10
- ^oDepartment of Oral Biology, University of Nebraska Medical Center 11
- [#]Address correspondence to: 12
- Thomas M. Petro, PhD 13
- Dept. of Oral Biology 14
- Univ. of Nebraska Med. Ctr. 15
- 40th and Holdrege St. 16
- Lincoln, NE 68583-0740 17
- phone: 402-472-1327 18
- 402-472-2551 19 fax:
- email: tpetro@unmc.edu 20
- 21
- 22
- 23 Short Running Title:
- Interleukin-6 and control of early TMEV infection 24
- 25 26 Word Count: 5,615
- 27 241 Words in Abstract
- 28 29 30 31
- 32
- 33
- 34
- 35
- 36 37
- JVI Accepts published online ahead of print

38	
39	
40	
41	
42 //3	
44	
45	
46	
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
54	
55	
56	
57	
58	
59	
60 61	
62	
63	
64	
65	
66	
07 68	
69	
70	
71	
72	
/3	

75 ABSTRACT

76

During Theiler's virus (TMEV) infection of macrophages it is thought that high IL-6 levels 77 contribute to demyelinating disease found in chronically infected SJL/J mice but absent 78 in B10.S mice capable of clearing the infection. Therefore, IL-6 expression was 79 measured in TMEV-susceptible SJL/J and TMEV-resistant B10.S macrophages during 80 their infection with TMEV DA strain or responses to LPS or poly I:C. Unexpectedly, IL-6 81 82 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 83 B10.S, SJL/J, and RAW264.7 macrophage cells, IL-6 expression was dependent on 84 extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) 85 and enhanced by exogenous IL-12. In SJL/J and RAW264.7 macrophages, exogenous 86 IL-6 resulted in decreased TMEV replication, earlier activation of STAT1 and STAT3, 87 production of nitric oxide, and earlier up-regulation of several anti-viral genes 88 89 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 90 addition, neutralization of endogenous IL-6 from SJL/J macrophages with Fab 91 antibodies did not exacerbate early TMEV infection. Therefore, endogenous IL-6 92 expression after TMEV infection is dependent on ERK MAPK, enhanced by IL-12, but 93 94 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 95 oxide production and anti-viral STAT1 activation. These results indicate that immediate-96 early production of IL-6 could protect macrophages from TMEV infection. 97

98

<u>NI Accepts published online ahead of print</u>

99 INTRODUCTION

Interleukin (IL)-6 is a pleiotropic cytokine expressed by many cell types that is induced 100 by microbes and other cytokines. IL-6 can play a beneficial role during immune 101 102 response by contributing to neutrophil activity, nitric oxide production and development 103 of the Th17 CD4 T cell subset, and a beneficial role in the brain (43) and neuronal 104 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 105 106 autoimmune responses (24). During microbial induction of IL-6, activation of p38 107 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 108 (ERK) MAPK signaling pathways contribute to IL-6 expression (21, 49) and stabilization of IL-6 mRNA (2) for protein translation. 109

Macrophages express IL-6 when they encounter microbes such as bacteria, 110 fungi, or viruses (44). While the neutrophil- and Th17-promoting activity of IL-6 is critical 111 in controlling certain bacterial or fungal infections, less is known about the role of IL-6 in 112 viral infections. Recently, an antiviral role for IL-6 has been shown in mouse poxvirus 113 infections, where early IL-6 production is required to control a potentially lethal infection 114 115 (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 116 viruses from infected macrophages could result in viral spread to other tissues due to 117 118 macrophage chemotaxis and could result in persistent IL-6 expression in affected tissue causing inflammatory pathologies and autoimmune diseases (16). Similarly, persistent 119 elevation of IL-6 is associated with the development of experimental autoimmune 120

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.

125 Theiler's murine encephalomyelitis virus (TMEV) causes an acute infection that is 126 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 127 128 prototypical TMEV-resistant strains whereas SJL/J mice fail to clear TMEV from macrophages and dendritic cells, resulting in persistent infection (25). Subsequent 129 130 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 131 132 shown that a week after infection with TMEV, CNS macrophages, microglial cells, and 133 astrocytes from SJL/J mice express IL-6 to a greater extent than C57BI/6 mice (14, 17, 34). A proposed theory is that chronic production of IL-6 in the CNS of persistently 134 infected SJL/J mice contributes to demyelinating disease in response to TMEV(17). 135 We propose that the dichotomy of TMEV persistence seen in resistant and 136

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

143	TMEV(37), yet this enhanced endogenous IFN β expression is insufficient to control
144	TMEV replication. However, pretreatment with additional exogenous recombinant $IFN\beta$
145	decreases TMEV replication in SJL/J macrophages to amounts seen in B10.S
146	macrophages (37). We found that p38 ERK MAPK pathways are activated in response
147	to TMEV infection of B10.S or SJL/J macrophages and play a role in IL-12 and IFN β
148	expression (37). Therefore, differences in MAPKs, IL-12, and IFN β may be related to
149	disparities in susceptibility of macrophages to persistent infection with TMEV.
150	In order to further understand the differences between SJL/J and B10.S mice that
151	could account for differences in TMEV persistence, we examined IL-6 expression in
152	macrophages during the early response to TMEV. Unexpectedly, we found that during
153	early response to infection with TMEV, B10.S macrophages expressed significantly
154	more IL-6 than SJL/J macrophages. Moreover, pre-treatment of macrophages with
155	exogenous IL-6 reduced TMEV replication but neutralization of endogenous TMEV-
156	induced IL-6 with Fab antibodies failed to affect TMEV replication in SJL/J
157	macrophages. Additionally, we show that TMEV-induced IL-6 secretion in
158	macrophages depends on ERK-MAPK and is enhanced by IL-12. However, neither IL-
159	6-induced nitric oxide production nor STAT1 activation are essential to IL-6 reducing
160	TMEV infection.

161 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

165	maintained in DMEM with 10% FBS with 50 μ g/mi gentamycin. The p38 MAP-kinase
166	inhibitor SB203580 and ERK MAPK inhibitor U0126 were obtained from Promega
167	Corporation (Madison, WI), E. coli LPS O127:B8 was obtained from Sigma Chemical
168	Co.(St. Louis, MO), and poly I:C was obtained from InvivoGen (San Diego, CA). The
169	inducible nitric oxide synthase inhibitor, L-NIL, was obtained from TOCRIS Bioscience
170	(Bristol, UK) and incubated with macrophages where indicated at 10 $\mu M.~$ The nitric
171	oxide donor spermine NONOate was obtained from Invitrogen (Carlsbad, CA) and
172	incubated with macrophages where indicated at 1 mM. Affinity purified neutralizing rat
173	antibody to mouse IL-6 (clone MP5-20F3) (48) was obtained from Invitrogen. Fab
174	fragments of neutralizing anti-IL-6 were obtained after papain digestion using the Fab
175	preparation kit of Pierce (Thermo Fisher Scientific, Rockford, IL). The initial stock of the
176	DA strain of TMEV was obtained from Dr. Kristen Drescher, Department of Medical
177	Microbiology and Immunology, Creighton University, Omaha, Nebraska. TMEV was
178	grown in BHK-21 cells. The titer of stock cultures of TMEV was 2.5×10^6 PFU/ml and
179	macrophages cultures were infected with 2.5 x 10^5 PFU of TMEV unless otherwise
180	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 1×10^6 cells/2 ml of DMEM cell culture medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen), and 50 µg/ml gentamycin (Invitrogen). After 24 h, nonadherent cells were removed and 1 ml of culture medium added. Adherent cells were

188	either untreated or pretreated for 30 min with 20 μM of SB203580, 40 μM U0126, 1
189	ng/ml IL-12 p70 (p35/p40; BD-Pharmingen, San Diego, CA), 10 ng/ml IFN eta
190	(Minneapolis, MN) or 10 ng/ml IL-12 p40 (BD-Pharmingen). Untreated or pretreated
191	macrophages were uninfected, infected with 100 μI of the TMEV stock (2.5 x 10^5 PFU),
192	stimulated with 1 μg /ml LPS, or stimulated with 50 μg poly I:C or left unstimulated. After
193	3, 8, or 24 h of infection or stimulation, supernatants were collected for ELISA and cell
194	extracts were collected for RNA preparation and quantitative RT-PCR.
195	RNA interference. Validated inhibitory siRNA targeting mouse STAT1 and
196	STAT3 was purchased from Cell Signaling, Inc. (Danvers, MA) and transfected into
197	RAW264.7 cells according to manufacturer's specifications using the nucleofection kit of
198	Amaxa 36 h prior to challenge with TMEV.
199	RNA preparation and quantitative RT-PCR. RNA was extracted from cells
200	using the RNAeasy kit of Qiagen (Valencia, CA), PerfectPure kit from 5Prime
201	(Gaithersburg, MD), or the Purelink kit from Ambion/Invitrogen (Carlsbad, CA) according
202	to the manufacturer's specifications. One-hundred ng to one μg of RNA was reverse
203	transcribed in 0.5 mM each of dATP, dGTP, dTTP, and dCTP, 20 U of RNAse inhibitor
204	with Superscript II reverse transcriptase (Invitrogen) at 42°C for 1.5 h followed by 94°C
205	for 5 min. One twenty-fifth of the cDNA sample was incubated with 0. 4 μM of the
206	following primer pairs (Invitrogen): IFN β sense 5' ATGAACAACAG GTGGATCCTCC 3'

greater than 90% Mac-1⁺ as determined by FACS analysis. These macrophages were

207 and anti-sense 5' AGGAGCTCCTGACATTTCCGAA 3'; IL-6 sense 5' ATGAAGTTCCT

8

208 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 antisense 5' TTCCCTATTTTCCGTGGCTGGG 3'; IRF-9 sense 5' ATGGCCTCA 211 GGCAAAGTACGCT 3' and antisense 5' TTCGCTTGCATGG TGATTTCTG 3'; TMEV 212 sense 5' CTTCCCATTC TACTGCAATG 3' and antisense 5' GTGTTCCTGG 213 214 TTTACAGTAG3'; or GAPDH sense 5'-TTGTCAGCAA TGCATCCTGCAC-3' and 215 antisense 5'-ACAGCTTTCCA GAGGGGCCATC-3'. Quantitative PCR reactions were run on an ABI Prism 7000 thermal cycler at 50 °C for 2 min, 95 °C for 10 min, 45 cycles 216 of 95 °C for 15 s/60 °C for 30 s. Cycle thresholds (Ct) of sample were normalized to Ct 217 of GAPDH for that sample (Δ Ct), and then normalized to the average Δ Ct of the control 218 samples ($\Delta\Delta$ Ct) after which data are expressed as relative levels of mRNA using 2^{- $\Delta\Delta$ Ct}. 219 When macrophages are pretreated, TMEV RNA data are reported as percentage of 220 221 TMEV RNA in non-treated but infected macrophages. ELISAs. ELISA plates were coated with antibodies to mouse IL-6 (MP5-222 20F3;BD-Pharmingen), the plates were blocked with PBS/10% FBS. After washes, cell 223 224 culture supernatants or serial dilutions of recombinant IL-6 (BD-Pharmingen) were

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

added to each well. IL-6 was measured by determining optical densities at OD 450 nm

wavelength with reference OD 570 nm using an ELISA spectrophotometric plate reader.

231	PAGE and western blot analysis. Cell lysates were obtained from RAW cells
232	challenged with TMEV with or without treatment with 10 ng/ml recombinant murine IL-6
233	(BD-Pharmingen, San Diego, CA) or murine recombinant IFN β (Interferon Source,
234	Piscataway, NJ). Twenty μI of each sample containing 20 μg of protein in sample buffer
235	with bromophenol blue was run on a 10% SDS, Tris-glycine- polyacrylamide gel and
236	transferred to a nitrocellulose membrane. The membrane was treated with blocking
237	buffer for 1 h at room temperature, followed by incubation in 1:500 dilution of rabbit IgG
238	anti-phospho-STAT1(Invitrogen, Camarillo, CA), 1:1000 dilution of anti-phospho-STAT3
239	(Cell Signalling, Beverly, MA), 1:500 dilution of anti-mouse STAT1(Invitrogen), 1:1000
240	dilution of anti-mouse STAT3 (Invitrogen), or 1:500 mouse anti-tubulin E7
241	(Developmental Studies Hybridoma Bank, University of Iowa) and then 1:5000 dilution
242	of IRDye® 800CWGoat Anti-Rabbit IgG (Rockland Immunochemicals, Inc., Gilbertsville,
243	PA) or Alexa Fluor680-labeled anti-Mouse IgG (Rockland Immunochemicals). The
244	washed membrane was scanned with a LICOR Odyssey® Infrared Imaging System and
245	densitometric analysis done with LICOR imaging software.
246	Nitric Oxide Assay. Induction of nitric oxide (NO) was assayed in culture
247	supernatants by measuring nitrite using the Greiss reagent kit of Invitrogen. Briefly, 20
248	μL of Griess Reagent was mixed with 150 μL of supernatant plus 130 μL of deionized
249	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 aspectrophotometer.

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.

255

256 **RESULTS**

257 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

260 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

262 challenge with TMEV. IL-6 mRNA was detected at 3 and 8 h after TMEV challenge

263 (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,

265 B10.S macrophages produced significantly more IL-6 protein 8 h after TMEV challenge

266 compared with SJL/J macrophages in response to TMEV (Fig. 1 B). Because TMEV

267 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

269 contribute to better control of TMEV replication.

270 To determine if exogenous IL-6 could impede TMEV replication in SJL/J

271 macrophages during early infection, recombinant IL-6 was added to SJL/J macrophages

272 30 min prior to TMEV infection and either left in the media during infection or washed

273 out by changing the media just prior to infection. Treatment with 10 ng/ml exogenous 274 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 275 IL-6 significantly reduced TMEV RNA at 24 h PI in SJL/J macrophages (Fig. 1D). 276 Equally significant, IL-6 preemptively triggered anti-viral activity in SJL macrophages 277 278 even when exogenous IL-6 was added for only 30 min and washed out prior to infection 279 (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 280 SJL/J macrophages. 281

282 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 283 284 was added to SJL/J macrophages at the time of TMEV infection (48). Surprisingly, 285 addition of either whole anti-IL-6 antibody or isotype control antibody both resulted in a 286 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 Fcy 287 288 receptors may also induce anti-viral immunity in macrophages. Indeed addition of 289 isotype antibody had a similar effect on TMEV infection of macrophages. Therefore we 290 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 291 292 decrease but also did not increase TMEV replication in infected SJL/J macrophages 293 (Fig. 1E).

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

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

309 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 310 infection of macrophages from both B10.S and SJL/J mice strongly activates ERK 311 MAPK and weakly activates p38 MAPK (37). Therefore, macrophages from B10.S and 312 313 SJL/J mice were pretreated with ERK and p38 MAPK inhibitors 30 min prior to TMEV 314 infection. Twenty-four h after infection, the amount of TMEV-induced IL-6 protein 315 secreted by B10.S macrophages was significantly greater than that secreted by SJL/J

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

326 We have also shown that the TLR3 pathway is involved in macrophage cytokine responses to TMEV infection by inducing IFN β , IL-12, and IL-23 (1). Therefore, we 327 compared the response of macrophages from each strain when treated with the TLR3 328 agonist, polyI:C or the TLR4 agonist, LPS. B10.S macrophages expressed significantly 329 more IL-6 mRNA and protein than SJL/J macrophages treated with LPS (Fig. 3 A, B) or 330 polyI:C (Fig. 3 C, D), but in macrophages from both strains the level of IL-6 expression 331 332 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 333 agonists (37), macrophages were also pretreated with U0126. Inhibition of ERK MAPK 334 335 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-6 336 337 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.

IL-6 expression by macrophages in response to TMEV is enhanced by IL-340 341 12. Previously, we showed that SJL/J macrophages responding to TMEV express significantly more IFN β and IL-12 p40 (p40/p40) but significantly less IL-12 p70 342 (p35/p40) than B10.S macrophages. In addition we showed that addition of IFN β , IL-12 343 p40, or p70 decreases TMEV replication in SJL/J macrophages (37). To see if these 344 cytokines could affect TMEV-induced IL-6, B10.S and SJL/J macrophages were 345 pretreated with the IL-12 p40 homodimer, bioactive IL-12 p70, or IFN β 30 min prior to 346 and during TMEV challenge. Treatment with IL-12 p70 or p40 significantly enhanced IL-347 348 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 349 upon pretreatment with IFN β was not significant (Fig. 4). As we have seen before, IL-350 351 12 and IFN β treatment reduced TMEV replication in both B10.S and SJL/J macrophages (Fig. 4C). Therefore deficient production of IL-12 during response to 352 353 TMEV could contribute to diminished IL-6 expression by SJL/J macrophages. 354 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 355 356 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, 357 RAW264.7 cells were treated with IL-12 with or without U0126 during TMEV infection. 358 Pretreatment with IL-12 enhanced IL-6 mRNA (Fig. 5 A) and protein (Fig. 5 B) 359

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. 364 365 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 366 exogenous recombinant IL-6 at 30 min prior to and during challenge with TMEV. Like 367 SJL/J macrophages, treatment of RAW264.7 cells with IL-6 significantly decreased 368 TMEV RNA at 24 h PI (Fig. 6 A). Interestingly, as little as 0.1 ng/ml significantly 369 reduced TMEV RNA 24 h PI, (Fig. 6B). Furthermore, IL-6 treatment enhanced IL-6 370 371 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 372 induced events occur in RAW264.7 are slightly different than that seen in SJL/J 373 macrophages. 374

IL-6 antiviral activity against TMEV replication is associated with activation
 of STAT1 related anti-viral activity. Like IFNβ, 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 IFNβ expression. To determine if IL-6 activates STAT1 and induces
 expression of IRFs that are downstream of STAT1 during TMEV infection of

382	macrophages, RAW264.7 cells were treated with IL-6, IFN β , or TMEV alone or in
383	combination and phospho-STAT1, as well as phoshpo-STAT3 were evaluated by
384	western blot. TMEV infection alone failed to activate STAT1 by 30 min PI but did so by
385	6 h PI (Fig. 7A). In contrast, treatment of RAW264.7 cells with either IL-6 or IFN eta
386	activated STAT1 and STAT3 as early as 30 min after TMEV challenge. To determine if
387	STAT1 activation is involved in control of TMEV replication, RAW264.7 cells were
388	transfected with small inhibitory RNA that has been verified to reduce STAT1 (siSTAT1)
389	or siSTAT3. Knockdown of STAT1 resulted in significantly increased TMEV RNA while
390	knockdown of STAT3 failed to affect TMEV RNA replication in RAW264.7 cells (Fig.
391	7B). However, pretreatment of STAT1-knocked-down RAW264.7 cells with exogenous
392	IL-6 resulted in a reduction of TMEV infection back to a level seen in RAW264.7 cells
393	infected with TMEV. Moreover, addition of exogenous IL-6 to STAT3-knocked down
394	and STAT1/STAT3 double-knocked down RAW264.7 cells significantly reduced TMEV
395	RNA replication compared with untreated RAW264.7 cells infected with TMEV (Fig. 7B).
396	Therefore, STAT1 activation contributes to control of TMEV replication and the
397	activation of STAT3 may negatively impact the antiviral effect of STAT1. Consistent
398	with STAT1 activation, treatment of RAW264.7 cells with IL-6 resulted in significant
399	enhancements of TMEV-induced IRF1 (Fig. 7C), IRF7 (Fig. 7D), IRF9 (Fig. 7E), and
400	IFN β (Fig. 7F). These results confirm that IL-6 can directly limit viral replication in
401	macrophages early after infection with TMEV by initiating a STAT1 anti-viral program.
402	IL-6 induces nitric oxide in RAW264.7 cells. IL-6 (42) and activated STAT1

403 (29) also induce expression of nitric oxide synthase and nitric oxide production, which is

 replication in macrophages by inducing nitric oxide. To explore this possibili challenged RAW264.7 cells were treated with 10 ng/ml IL-6 in the presence of L-NIL, an inducible nitric oxide synthase inhibitor (27), and production of n was measured. TMEV infection of RAW264.7 cells did not result in significat oxide production at 24 h Pl (see Fig. S3A). However, addition of exogenous TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide de repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	04 a	a potent anti-viral factor (41). Therefore, another possibility is that IL-6 controls TMEV
 challenged RAW264.7 cells were treated with 10 ng/ml IL-6 in the presence of L-NIL, an inducible nitric oxide synthase inhibitor (27), and production of n was measured. TMEV infection of RAW264.7 cells did not result in significat oxide production at 24 h PI (see Fig. S3A). However, addition of exogenous TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide de repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	05 I	replication in macrophages by inducing nitric oxide. To explore this possibility, TMEV-
 of L-NIL, an inducible nitric oxide synthase inhibitor (27), and production of n was measured. TMEV infection of RAW264.7 cells did not result in significat oxide production at 24 h PI (see Fig. S3A). However, addition of exogenous TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide de repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	06 0	challenged RAW264.7 cells were treated with 10 ng/ml IL-6 in the presence or absence
 was measured. TMEV infection of RAW264.7 cells did not result in significat oxide production at 24 h PI (see Fig. S3A). However, addition of exogenous TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide de repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	07 0	of L-NIL, an inducible nitric oxide synthase inhibitor (27), and production of nitric oxide
 oxide production at 24 h PI (see Fig. S3A). However, addition of exogenous TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide de repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	08 \	was measured. TMEV infection of RAW264.7 cells did not result in significant nitric
TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide de repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro.	09 (oxide production at 24 h PI (see Fig. S3A). However, addition of exogenous IL-6 to
 secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide de repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	10 -	TMEV infected RAW264.7 cell resulted in a significant enhancement of nitric oxide
 repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L- reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	11 \$	secretion, which was prevented by L-NIL. IL-6 or NONOate, a nitric oxide donor,
 reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, w induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	12 I	repressed TMEV replication in RAW264.7 cells (see Fig. S3B). However, L-NIL did not
 induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive IL-6-induced antiviral effect observed in macrophages in vitro. 	13 I	reverse the IL-6 repression of TMEV replication in RAW264.7 cells. Thus, while IL-6
IL-6-induced antiviral effect observed in macrophages in vitro.	14 i	induces nitric oxide and nitric oxide is anti-viral, it does not play the decisive role in the
	15 l	IL-6-induced antiviral effect observed in macrophages in vitro.

417 **DISCUSSION**

The data herein show that IL-6 protects macrophages early after infection with TMEV by 418 419 decreasing virus replication in macrophages. These results are somewhat surprising because research on chronic TMEV infection has supported the theory that enhanced 420 IL-6 may play a detrimental role in the immunopathology of TMEV in susceptible mice 421 (14). Indeed, a recent report has shown that SJL/J mice infected with TMEV exhibit 422 423 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 424 425 infection. We show herein that the antiviral effect of IL-6 takes place within the first 6 h

426	after infection. Therefore the beneficial effect of IL-6 is early after TMEV infection, but
427	not later. It is also possible that TMEV-resistant C57BI/6 mice may have a different
428	mechanism to control TMEV infection in macrophages compared with TMEV-resistant
429	B10.S mice. The results herein indicate that during the first day after infection in vitro,
430	IL-6 protein production by macrophages from TMEV-resistant B10.S mice is greater
431	than from macrophages of TMEV-susceptible SJL/J and this difference may play an
432	important role in determining the severity of the infection. Moreover, significant levels of
433	IL-6 are not produced until 8 h after TMEV infection and IL-6 is only effective at
434	controlling TMEV replication during the first 8 h after infection. Therefore, IL-6 produced
435	by the TMEV infected macrophages helps to protect uninfected macrophages from
436	subsequent infection. Altogether, the present data suggest the CNS demyelination that
437	SJL/J mice develop several weeks to months after infection with TMEV may be due in
438	part to an inability of SJL/J macrophages to control viral replication very early after the
439	initial infection. Similarly, B10.S mice that do not develop demyelination following
440	infection with TMEV may stem from better early control of TMEV replication in
441	macrophages. Our results indicate that ability to control TMEV replication in
442	macrophages begins during the first 24h after infection is to a large extent dependent on
443	macrophage production of IL-6, which then protects uninfected cells from infection.
444	Thus, IL-6 production by virally infected cells may have dual roles in viral infection by
445	contributing to both anti-viral immunity and subsequent pathology.
446	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

449 with TMEV. Other reports have shown that IL-6 is involved in neuronal health facilitating neuronal differentiation, neurite outgrowth, survival, regeneration (47) and 450 oligodendrocyte differentiation (52). In stark contrast to these reports are others which 451 have suggested that chronic expression of IL-6 in TMEV-susceptible SJL/J mice is 452 responsible for development of demyelinating disease (17). Hou et al. (14) showed that 453 IL-6 levels in the brain and spinal cord of SJL/J mice are elevated 8-90 days post 454 455 infection compared with TMEV resistant C57BI/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 456 replication, thereby rendering these mice susceptible to chronic viral infection and a 457 chronically elevated IL-6 response. Alternatively, enhanced IL-6 in the CNS of TMEV 458 infected SJL/J mice may not be a reflection of a heightened IL-6 response of infected 459 cells to TMEV but may be due to enhanced infiltration of macrophages into the CNS, 460 461 which then are infected with TMEV and produce IL-6.

The anti-viral and pathological effects of IL-6 notwithstanding, the signaling 462 pathways that lead to IL-6 expression in response to TMEV remain unclear. Previous 463 reports showed that ERK and p38 MAPK pathways are involved in both innate anti-viral 464 immunity (7) and IL-6 expression (49). The results herein confirm that activation of ERK 465 466 MAPKs contributes to IL-6 expression in response to TMEV. However, maximum ERK 467 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 468 macrophages following TMEV infection (37, 38). Activated ERK MAPKs have been 469 shown to phosphorylate downstream transcription factors such as the cyclic AMP 470 response element binding protein (CREB) at serine 133 (4, 51, 45), CREB response 471

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.

476 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 477 of Th1 development during adaptive immune responses (15). In addition, our results 478 479 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 480 significant role in IL-6 expression during viral infection (18), IL-6 expression depends in 481 part on activation of ERK (19), and that IL-12 activates ERK-MAPK (20). These results 482 483 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 484 (37). Therefore, IL-12 expression by macrophages that is responsible for Th1 485 development during adaptive immune responses also contributes to IL-6 expression 486 487 during innate immune responses, both roles of IL-12 are likely to be beneficial to early 488 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

494 inducer of iNOS and nitric oxide production (31), which has anti-viral properties (41). 495 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-6-496 induced nitric oxide production did not reverse the anti-viral effect of IL-6. This is most 497 likely because IL-6 also activated STAT1 and STAT3 that lead to the induction of 498 STAT1-downstream anti-viral genes, IRF1, IRF7, and IRF9. Therefore the data suggest 499 500 that early after infection with TMEV, IL-6 controls acute viral infection by inducing 501 multiple innate antiviral programs.

502 One of the discrepancies noted herein is that between IL-6 mRNA expression 503 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, 504 505 which is especially notable in the TMEV-susceptible SJL/J macrophages. At 24 and 28 506 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 507 regulatory protein (KHSRP) was shown to target the AU-rich elements in the IL-6 mRNA 508 509 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-6 510 511 mRNA without affecting IL-6 mRNA (50). Our results suggest that differences in 512 translational or inhibitory control of IL-6 expression following infection of macrophages could contribute to susceptibility versus resistance of macrophages to TMEV. 513

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

516	disease outcomes between B10.S and SJL/J mice. Whereas B10.S mice with
517	enhanced early IL-6 are capable of clearing TMEV, in SJL/J mice TMEV replication is
518	not reduced by IL-6 unless exogenous IL-6 is added, suggesting that these mice do not
519	produce IL-6 quickly enough or in sufficient quantity to control viral replication. The
520	present investigation suggests that enhanced early expression of IL-6 by macrophages
521	during infection with a macrophage-trophic virus, TMEV, is responsible for better control
522	of viral replication in TMEV-resistant B10.S macrophages compared with TMEV-
523	susceptible SJL/J macrophages. These results indicate that the persistent TMEV
524	infection of macrophages in the CNS of SJL/J mice could be the result of insufficient IL-
525	6 production that controls early TMEV infection through multiple anti-viral mechanisms
526	that include nitric oxide production and STAT1 activation. Furthermore, the present
527	data clearly shows that IL-12 and ERK MAPK play a major role in TMEV-induced IL-6.
528	While ERK MAPK is required for IL-6 expression in response to TMEV its activation is
529	equivalent in both strains. In contrast our previous studies point to differences in TLR
530	signaling and IL-12 expression that may contribute to strain differences in TMEV-
531	induced IL-6. These results are supported by our data showing that TMEV-induced IL-6
532	is also antiviral in RAW264.7 cells, IL-6 expression is enhanced by IL-12, and IL-6 leads
533	to activation of STAT1, expression of its downstream genes IRF1, IRF7, IRF 9, and IFN-
534	beta, as well as induction of nitric oxide. These results are significant because
535	persistent infection of SJL/J macrophages by TMEV contributes to the inflammatory
536	autoimmune demyelination in the CNS of TMEV infected SJL/J mice, which mimics
537	human Multiple Sclerosis, that is not seen in TMEV infected B10.S mice. It remains to
538	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
- 541 chronic viral infections.

543 ACKNOWLEDGEMENTS

This work was supported by funding from the University of Nebraska Medical Center
College of Dentistry and University of Nebraska Lincoln, School of Biological Sciences,
and supported by Award Number 8P30GM10350903 and 5P20GM103489 from the
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
does not necessarily represent the official views of the National Center for Research
Resources or the National Institutes of Health.

551

552 **REFERENCES**

553

- AI-Salleeh, F., and T. M. Petro. 2007. TLR3 and TLR7 are involved in expression of IL-23 subunits while TLR3 but not TLR7 is involved in expression of IFN-beta by Theiler's virus-infected RAW264.7 cells. Microbes Infect 9:1384-92.
 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
 interleukin-17 plus tumor necrosis factor-alpha-induced stabilization of

- interleukin-6 mRNA in human pancreatic myofibroblasts. Biochim Biophys Acta
 1591:69-74.
 3. Banerjee, S., K. Narayanan, T. Mizutani, and S. Makino. 2002. Murine
 coronavirus replication-induced p38 mitogen-activated protein kinase activation
 promotes interleukin-6 production and virus replication in cultured cells. J Virol
 76:5937-48.
- 4. Cammarota, M., L. R. Bevilaqua, P. R. Dunkley, and J. A. Rostas. 2001.
- Angiotensin II promotes the phosphorylation of cyclic AMP-responsive element
 binding protein (CREB) at Ser133 through an ERK1/2-dependent mechanism. J
 Neurochem **79:**1122-8.
- 571 5. Clatch, R. J., S. D. Miller, R. Metzner, M. C. Dal Canto, and H. L. Lipton.

572 1990. Monocytes/macrophages isolated from the mouse central nervous system
573 contain infectious Theiler's murine encephalomyelitis virus (TMEV). Virology
574 **176**:244-54.

- 575 6. Dhamija, S., N. Kuehne, R. Winzen, A. Doerrie, O. Dittrich-Breiholz, B. K.
 576 Thakur, M. Kracht, and H. Holtmann. 2011. Interleukin-1 activates synthesis of
- 577 interleukin-6 by interfering with a KH-type splicing regulatory protein (KSRP)-
- 578 dependent translational silencing mechanism. J Biol Chem **286**:33279-88.
- 579 7. Dong, C., R. J. Davis, and R. A. Flavell. 2002. MAP kinases in the immune
 580 response. Annu Rev Immunol 20:55-72.
- 8. Droogmans, L., I. Cludts, Y. Cleuter, R. Kettmann, and A. Burny. 1992.
- 582 Nucleotide sequence of the bovine interleukin-6 gene promoter. DNA Seq 3:115-
- 583

7.

584	9.	Gadient, R. A., and U. Otten. 1994. Identification of interleukin-6 (IL-6)-
585		expressing neurons in the cerebellum and hippocampus of normal adult rats.
586		Neurosci Let 182:243-246.
587	10.	Grassi, C., B. Luckow, D. Schlondorff, and U. Dendorfer. 1999.
588		Transcriptional regulation of the interleukin-6 gene in mesangial cells. J Am Soc
589		Nephrol 10: 1466-77.
590	11.	Hama, T., M. Miyamoto, H. Tsukui, C. Nishio, and H. Hatanaka. 1989.
591		Interleukin-6 as a neurotrophic factor for promoting the survival of cultured basal
592		forebrain cholinergic neurons from postnatal rats. Neurosci Lett 104: 340-4.
593	12.	Harker, J. A., G. M. Lewis, L. Mack, and E. I. Zuniga. 2011. Late Interleukin-6
594		Escalates T Follicular Helper Cell Responses and Controls a Chronic Viral
595		Infection. Science 334:825-829.
596	13.	Hemmann, U., C. Gerhartz, B. Heesel, J. r. Sasse, G. n. Kurapkat, J.
597		Grotzinger, A. Wollmer, Z. Zhong, J. E. D. Jr, L. Graeve, P. C. Heinrich, and
598		F. Horn. 1996. Differential Activation of Acute Phase Response Factor/Stat3 and
599		Stat1 via the Cytoplasmic Domain of the Interleukin 6 Signal Transducer gp130.
600		J Biol Chem 271 :12999-13007.
601	14.	Hou, W., H. S. Kang, and B. S. Kim. 2009. Th17 cells enhance viral persistence
602		and inhibit T cell cytotoxicity in a model of chronic virus infection. J Exp Med
603		206: 313-28.
604	15.	Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M.
605		Murphy. 1993. Development of TH1 CD4+ T cells through IL-12 produced by
606		Listeria-induced macrophages. Science 260:547-9.

JVI Accepts published online ahead of print

607	16.	Ishihara, K., and T. Hirano. 2002. IL-6 in autoimmune disease and chronic
608		inflammatory proliferative disease. Cytokine Growth Factor Rev 13:357-68.
609	17.	Jin, YH., M. Mohindru, M. H. Kang, A. C. Fuller, B. Kang, D. Gallo, and B. S.
610		Kim. 2007. Differential Virus Replication, Cytokine Production, and Antigen-
611		Presenting Function by Microglia from Susceptible and Resistant Mice Infected
612		with Theiler's Virus. J Virol 81:11690-11702.
613	18.	Kapil, P., R. Atkinson, C. Ramakrishna, D. J. Cua, C. C. Bergmann, and S. A.
614		Stohlman. 2009. Interleukin-12 (IL-12), but not IL-23, deficiency ameliorates viral
615		encephalitis without affecting viral control. J Virol 83:5978-86.
616	19.	Kim, S. H., J. Kim, and R. P. Sharma. 2004. Inhibition of p38 and ERK MAP
617		kinases blocks endotoxin-induced nitric oxide production and differentially
618		modulates cytokine expression. Pharmacol Res 49:433-439.
619	20.	Kondadasula, S. V., J. M. Roda, R. Parihar, J. Yu, A. Lehman, M. A. Caligiuri,
620		S. Tridandapani, R. W. Burry, and W. E. Carson. 2008. Colocalization of the
621		IL-12 receptor and $Fc\gamma RIIIa$ to natural killer cell lipid rafts leads to activation of
622		ERK and enhanced production of interferon- γ . Blood 111: 4173-4183.
623	21.	Leonard, M., M. P. Ryan, A. J. Watson, H. Schramek, and E. Healy. 1999.
624		Role of MAP kinase pathways in mediating IL-6 production in human primary
625		mesangial and proximal tubular cells. Kidney Int 56:1366-77.
626	22.	Li, X., S. Leung, S. Qureshi, J. E. Darnell, Jr., and G. R. Stark. 1996.
627		Formation of STAT1-STAT2 heterodimers and their role in the activation of IRF-1
628		gene transcription by interferon-alpha. J Biol Chem 271: 5790-4.

629	23.	Marie, I., J. E. Durbin, and D. E. Levy. 1998. Differential viral induction of
630		distinct interferon-alpha genes by positive feedback through interferon regulatory
631		factor-7. Embo J 17: 6660-9.
632	24.	Mihara, M., M. Hashizume, H. Yoshida, M. Suzuki, and M. Shiina. 2011. IL-
633		6/IL-6 receptor system and its role in physiological and pathological conditions.
634		Clin Sci (Lond) 122: 143-59.
635	25.	Monteyne, P., F. Bihl, F. Levillayer, M. Brahic, and J. F. Bureau. 1999. The
636		Th1/Th2 balance does not account for the difference of susceptibility of mouse
637		strains to Theiler's virus persistent infection. J Immunol 162:7330-4.
638	26.	Moore, T. C., F. M. Al-Salleeh, D. M. Brown, and T. M. Petro. 2011. IRF3
639		polymorphisms induce different innate anti-Theiler's virus immune responses in
640		RAW264.7 macrophages. Virology 418:40-8.
641	27.	Moore, W. M., R. K. Webber, G. M. Jerome, F. S. Tjoeng, T. P. Misko, and M.
642		G. Currie. 1994. L-N6-(1-Iminoethyl)lysine: A Selective Inhibitor of Inducible
643		Nitric Oxide Synthase. J Med Chem 37:3886-3888.
644	28.	Mott, K. R., D. Underhill, S. L. Wechsler, T. Town, and H. Ghiasi. 2009. A role
645		for the JAK-STAT1 pathway in blocking replication of HSV-1 in dendritic cells and
646		macrophages. Virol J 6:56.
647	29.	Nishiya, T., T. Uehara, H. Edamatsu, Y. Kaziro, H. Itoh, and Y. Nomura. 1997.
648		Activation of Stat1 and subsequent transcription of inducible nitric oxide synthase
649		gene in C6 glioma cells is independent of interferon-Î ³ -induced MAPK activation
650		that is mediated by p21ras. FEBS Letters 408: 33-38.

JVI Accepts published online ahead of print

651	30.	O'Gorman, W. E., P. Sampath, E. F. Simonds, R. Sikorski, M. O'Malley, P. O.
652		Krutzik, H. Chen, V. Panchanathan, G. Chaudhri, G. Karupiah, D. B. Lewis,
653		S. H. Thorne, and G. P. Nolan. 2010. Alternate mechanisms of initial pattern
654		recognition drive differential immune responses to related poxviruses. Cell Host
655		Microbe 8: 174-85.
656	31.	Oh, Y. S., YJ. Lee, E. Y. Park, and HS. Jun. 2011. Interleukin-6 treatment
657		induces beta-cell apoptosis via STAT-3-mediated nitric oxide production.
658		Diabetes/Metabolism Research and Reviews 27:813-819.
659	32.	Okuda, Y., S. Sakoda, C. Bernard, H. Fujimura, Y. Saeki, T. Kishimoto, and
660		T. Yanagihara. 1998. IL-6-deficient mice are resistant to the induction of
661		experimental autoimmune encephalomyelitis provoked by myelin oligodendrocyte
662		glycoprotein. Int. Immunol. 10:703-708.
663	33.	Oleszak, E. L., J. R. Chang, H. Friedman, C. D. Katsetos, and C. D.
664		Platsoucas. 2004. Theiler's virus infection: a model for multiple sclerosis. Clin
665		Microbiol Rev 17: 174-207.
666	34.	Palma, J. P., D. Kwon, N. A. Clipstone, and B. S. Kim. 2003. Infection with
667		Theiler's Murine Encephalomyelitis Virus Directly Induces Proinflammatory
668		Cytokines in Primary Astrocytes via NF-{kappa}B Activation: Potential Role for
669		the Initiation of Demyelinating Disease. J. Virol. 77:6322-6331.
670	35.	Pavelko, K. D., C. L. Howe, K. M. Drescher, J. D. Gamez, A. J. Johnson, T.
671		Wei, R. M. Ransohoff, and M. Rodriguez. 2003. Interleukin-6 Protects Anterior
672		Horn Neurons from Lethal Virus-Induced Injury. J Neurosci 23:481-492.

673	36.	Persson, E., O. S. Voznesensky, Y. F. Huang, and U. H. Lerner. 2005.
674		Increased expression of interleukin-6 by vasoactive intestinal peptide is
675		associated with regulation of CREB, AP-1 and C/EBP, but not NF-kappaB, in
676		mouse calvarial osteoblasts. Bone 37:513-29.
677	37.	Petro, T. M. 2005. Disparate expression of IL-12 by SJL/J and B10.S
678		macrophages during Theiler's virus infection is associated with activity of TLR7
679		and mitogen-activated protein kinases. Microbes Infect 7:224-32.
680	38.	Petro, T. M. 2005. ERK-MAP-kinases differentially regulate expression of IL-23
681		p19 compared with p40 and IFN-beta in Theiler's virus-infected RAW264.7 cells.
682		Immunol Lett 97:47-53.
683	39.	Rodriguez, M., K. D. Pavelko, C. W. McKinney, and J. L. Leibowitz. 1994.
684		Recombinant human IL-6 suppresses demyelination in a viral model of multiple
685		sclerosis. J Immunol 153: 3811-3821.
686	40.	Samoilova, E. B., J. L. Horton, B. Hilliard, TS. T. Liu, and Y. Chen. 1998. IL-
687		6-Deficient Mice Are Resistant to Experimental Autoimmune Encephalomyelitis:
688		Roles of IL-6 in the Activation and Differentiation of Autoreactive T Cells. J
689		Immunol 161: 6480-6486.
690	41.	Sanders, S. P., E. S. Siekierski, J. D. Porter, S. M. Richards, and D. Proud.
691		1998. Nitric Oxide Inhibits Rhinovirus-Induced Cytokine Production and Viral
692		Replication in a Human Respiratory Epithelial Cell Line. J Virol 72:934-942.
693	42.	Sawada, T., L. A. Falk, P. Rao, W. J. Murphy, and D. H. Pluznik. 1997. IL-6
694		induction of protein-DNA complexes via a novel regulatory region of the inducible
	673 674 675 676 677 678 679 680 681 682 683 684 683 684 685 684 685 684 685 684 685 688 687 688 689 690 691 692 693 694	673 36. 674

69	96		158: 5267-76.
69	97 4	43.	Schobitz, B., E. R. de Kloet, W. Sutanto, and F. Holsboer. 1993. Cellular
69	98		localization of interleukin 6 mRNA and interleukin 6 receptor mRNA in rat brain.
69	99		Eur J Neurosci 5: 1426-35.
70	00 4	44.	Sehgal, P. B., D. C. Helfgott, U. Santhanam, S. B. Tatter, R. H. Clarick, J.
70)1		Ghrayeb, and L. T. May. 1988. Regulation of the acute phase and immune
70)2		responses in viral disease. Enhanced expression of the beta 2-
70)3		interferon/hepatocyte-stimulating factor/interleukin 6 gene in virus-infected
70)4		human fibroblasts. J Exp Med 167: 1951-1956.
70)5 4	45.	Shaywitz, A. J., and M. E. Greenberg. 1999. CREB: a stimulus-induced
70	06		transcription factor activated by a diverse array of extracellular signals. Annu Rev
70)7		Biochem 68: 821-61.
70)8 4	46.	Shresta, S., K. L. Sharar, D. M. Prigozhin, H. M. Snider, P. R. Beatty, and E.
70)9		Harris. 2005. Critical Roles for Both STAT1-Dependent and STAT1-Independent
71	10		Pathways in the Control of Primary Dengue Virus Infection in Mice. J Immunol
71	1		175 :3946-3954.
71	2 4	47.	Spooren, A., K. Kolmus, G. Laureys, R. Clinckers, J. De Keyser, G.
71	13		Haegeman, and S. Gerlo. 2011. Interleukin-6, a mental cytokine. Brain Res Rev
71	4		67: 157-183.
71	15 4	48.	Starnes, H. F., Jr., M. K. Pearce, A. Tewari, J. H. Yim, J. C. Zou, and J. S.

nitric oxide synthase gene promoter: role of octamer binding proteins. J Immunol

716 **Abrams.** 1990. Anti-IL-6 monoclonal antibodies protect against lethal

717		Escherichia coli infection and lethal tumor necrosis factor-alpha challenge in
718		mice. J Immunol 145: 4185-91.
719	49.	Tuyt, L. M., W. H. Dokter, K. Birkenkamp, S. B. Koopmans, C. Lummen, W.
720		Kruijer, and E. Vellenga. 1999. Extracellular-regulated kinase 1/2, Jun N-
721		terminal kinase, and c-Jun are involved in NF-kappa B-dependent IL-6
722		expression in human monocytes. J Immunol 162: 4893-902.
723	50.	Xu, Z., SB. Xiao, P. Xu, Q. Xie, L. Cao, D. Wang, R. Luo, Y. Zhong, HC.
724		Chen, and LR. Fang. 2011. miR-365, a Novel Negative Regulator of
725		Interleukin-6 Gene Expression, Is Cooperatively Regulated by Sp1 and NF-kB. J
726		Biol Chem 286: 21401-21412.
727	51.	Zanassi, P., M. Paolillo, A. Feliciello, E. V. Avvedimento, V. Gallo, and S.
728		Schinelli. 2001. cAMP-dependent protein kinase induces cAMP-response
729		element-binding protein phosphorylation via an intracellular calcium
730		release/ERK-dependent pathway in striatal neurons. J Biol Chem 276:11487-95.
731	52.	Zhang, PL., M. Izrael, E. Ainbinder, L. Ben-Simchon, J. Chebath, and M.
732		Revel. 2006. Increased myelinating capacity of embryonic stem cell derived
733		oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6
734		receptor fusion protein. Mol Cell Neurosci 31:387-398.
735 736		
737		

	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),
rìn	743	0.01–10 ng/ml rec IL-6 (D), 3 μ g/ml anti-IL-6-Fab (E), or SJL/J macrophages were
d ło	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
ed	746	PI (F). RNA was reverse transcribed and relative levels of IL-6 mRNA (A) was evaluated
ah	747	by real-time PCR, IL-6 protein by ELISA (B), and TMEV by real-time PCR (C, D, E,F).
ne	748	Data are means of 3-5 samples per time point evaluated by the Student t test; *
onli	749	indicates <i>p</i> ≤ 0.05, ** p≤ 0.01.
hed	750	
lisl	751	FIG 2 ERK MAPK activation is required for optimum TMEV-induced IL-6 expression. 1
puk	752	x 10^6 macrophages were untreated (nil or control) or pretreated with 20 μM SB203580
1S	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

n before infection with 2 x 10⁵ PFU of TMEV. After 24 evaluated by real-time PCR (A), IL-6 protein secretion evaluated by ELISA (B), and TMEV RNA by real-time PCR (C). Data are 755 means of 5 samples from two experiments evaluated by the Student t test. * with 756 brackets indicates $p \leq 0.05$. 757

758

VI Acc

759

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. 1×10^{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 polyI: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; $p \leq 0.05$.

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. 1×10^{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 <i>t</i> test. * indicates comparisons considered significantly different; $p \le 0.05$.
776	

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 1×10^6 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.

FIG 6 IL-6 constrains TMEV replication during infection of RAW264.7 macrophages. 1 x 10⁶RAW264.7 macrophage cells were untreated (control) or treated with 10ng/ml IL-6 (A, C) or 0.01- 10 ng/ml IL-6 (B) during infection with 1 x 10⁶ PFU of TMEV. After 3, 6 (D), or 24 h (A, B, C) of infection relative levels of TMEV (A, B, D) or IL-6 (C) mRNA were evaluated by real-time PCR. Means are of 6 samples from two experiments each 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 macroph	hages. (A) Western blots of
				0 ()

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.

- (B) TMEV RNA measured by qRT-PCR in RAW264.7 cells transfected with siSTAT1-
- 800 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 IFN β (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 t test. * indicates means
804	considered significantly different; $p \leq 0.05$; n.d.= not detectable.
805	
806	
807	
808	
809	
810	
811	
812	
813	
814	
815	
816	
817	
818	

Figure 1. Moore et al.

820







Figure 4. Moore et al.





Figure 5. Moore et al.



Figure 6. Moore et al.





Figure 7. Moore et al.



JVI Accepts published online ahead of print