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

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Abbreviations:

IFN=interferon

IRF=interferon response factor

TMEV=Theiler's murine encephalomyelitis virus

MAPK=mitogen-activated protein kinase

ERK= extracellular signal-regulated kinase

EAE = experimental autoimmune encephalomyelitis

ABSTRACT

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 immediate-early 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 IFNβ expression is insufficient to control TMEV replication. However, pretreatment with additional exogenous recombinant IFNβ 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 IFNβ expression (37). Therefore, differences in MAPKs, IL-12, and IFNβ 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

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 10^6 PFU/ml and macrophages cultures were infected with 2.5 x 10^5 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 μg/ml gentamycin (Invitrogen). After 24 h, non-adherent cells were removed and 1 ml of culture medium added. Adherent cells were

187 greater than 90% Mac-1$^+$ as determined by FACS analysis. These macrophages 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 IFNβ
190 (Minneapolis, MN) or 10 ng/ml IL-12 p40 (BD-Pharmingen). Untreated or pretreated
191 macrophages were uninfected, infected with 100 μl of the TMEV stock (2.5 x 10^5 PFU),
192 stimulated with 1 μg/ml LPS, or stimulated with 50μg 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^{o}C for 1.5 h followed by 94^{o}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): IFNβ sense 5' ATGAACAACAG GTGGATCCTCC 3'
207 and anti-sense 5' AGGAGCTCCTGACATTTCCGAA 3'; IL-6 sense 5′ ATGAAGTTCCT
208 CTCTGCAAGAGACT 3' and antisense 5′ CACTAGGTTTGCC GAGTAGATCTC 3′;

IRF1 sense 5' ATGCCAATCACTCGAATGCGGA 3' and antisense 5' GGCTGC CACTCAGACTGTTCAA 3'; IRF-7 sense 5′ CCAGCGAGTGC TGTTTGGAGAC 3′ and antisense 5′ TTCCCTATTTTCCGTGGCTGGG 3′; IRF-9 sense 5' ATGGCCTCA GGCAAAGTACGCT 3' and antisense 5' TTCGCTTGCATGG TGATTTCTG 3'; TMEV sense 5' CTTCCCATTC TACTGCAATG 3' and antisense 5' GTGTTCCTGG TTTACAGTAG3'; or GAPDH sense 5'-TTGTCAGCAA TGCATCCTGCAC-3' and 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 of 95 °C for 15 s/60 °C for 30 s. Cycle thresholds (Ct) of sample were normalized to Ct of GAPDH for that sample (ΔCt), and then normalized to the average ΔCt of the control samples (ΔΔCt) after which data are expressed as relative levels of mRNA using $2^{-\Delta\Delta Ct}$. When macrophages are pretreated, TMEV RNA data are reported as percentage of TMEV RNA in non-treated but infected macrophages.

ELISAs. ELISA plates were coated with antibodies to mouse IL-6 (MP5-20F3;BD-Pharmingen), the plates were blocked with PBS/10% FBS. After washes, cell 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-Pharmingen) was added to each well. After 1 h, strepavidin horseradish peroxidase (1:1000; BD-Pharmingen) was added for 30 min and then 3,3', 5, 5' 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.

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 IFNβ (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 µL of Griess Reagent was mixed with 150 µL of supernatant plus 130 µL 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.

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

255

256 **RESULTS**

257 **TMEV-induced IL- 6 production is greater in TMEV-resistant B10.S macrophages.**
258 IL-6 contributes to both antiviral immunity and virus-induced pathology (3, 41). TMEV is
259 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-
261 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
264 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
268 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

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).

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 IFNβ 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 IFNβ 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**
309 **MAPK.** The cell signaling pathway activated by TMEV infection leading to IL-6
310 expression in macrophages is not well understood. We previously showed that TMEV
311 infection of macrophages from both B10.S and SJL/J mice strongly activates ERK
312 MAPK and weakly activates p38 MAPK (37). Therefore, macrophages from B10.S and
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 mRNA 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
327 responses to TMEV infection by inducing IFNβ, IL-12, and IL-23 (1). Therefore, we
328 compared the response of macrophages from each strain when treated with the TLR3
329 agonist, polyI:C or the TLR4 agonist, LPS. B10.S macrophages expressed significantly
330 more IL-6 mRNA and protein than SJL/J macrophages treated with LPS (Fig. 3 A, B) or
331 polyI:C (Fig. 3 C, D), but in macrophages from both strains the level of IL-6 expression
332 in response to poly I:C was substantially less than that in response to LPS or TMEV.
333 Because ERK MAPK is activated by both TLR3 agonists (see Fig. S2) and TLR4
334 agonists (37), macrophages were also pretreated with U0126. Inhibition of ERK MAPK
335 activation with U0126 significantly reduced IL-6 expression to the same extent in both
336 B10.S and SJL/J macrophages responding to LPS and significantly decreased IL-6
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-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)

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

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

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

macrophages, RAW264.7 cells were treated with IL-6, IFNβ, 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 IFNβ 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 IFNβ (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.

IL-6 induces nitric oxide in RAW264.7 cells. IL-6 (42) and activated STAT1 (29) also induce expression of nitric oxide synthase and nitric oxide production, which is

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

416

417 **DISCUSSION**

418 The data herein show that IL-6 protects macrophages early after infection with TMEV by
419 decreasing virus replication in macrophages. These results are somewhat surprising
420 because research on chronic TMEV infection has supported the theory that enhanced
421 IL-6 may play a detrimental role in the immunopathology of TMEV in susceptible mice
422 (14). Indeed, a recent report has shown that SJL/J mice infected with TMEV exhibit
423 greater levels of IL-6 in the CNS than TMEV-resistant C57Bl/6 mice starting at day 8
424 after infection (14). However, in that report IL-6 was not measured until day-8 after
425 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

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

462 The anti-viral and pathological effects of IL-6 notwithstanding, the signaling
463 pathways that lead to IL-6 expression in response to TMEV remain unclear. Previous
464 reports showed that ERK and p38 MAPK pathways are involved in both innate anti-viral
465 immunity (7) and IL-6 expression (49). The results herein confirm that activation of ERK
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
468 noticed differences in the intensity of ERK activation between SJL and B10.S
469 macrophages following TMEV infection (37, 38). Activated ERK MAPKs have been
470 shown to phosphorylate downstream transcription factors such as the cyclic AMP
471 response element binding protein (CREB) at serine 133 (4, 51, 45), CREB response

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

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

489 Overall the data here suggest a direct, interferon-like role for IL-6 in the
490 suppression of TMEV replication, which is exemplified by its ability to activate STAT1
491 and induce expression of STAT1 dependent genes. Indeed early reports regarding IL-
492 6, which was originally called Interferon-beta 2, showed it to have anti-viral activity (44).
493 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
496 and that nitric oxide does indeed control TMEV replication. However, preventing IL-6-
497 induced nitric oxide production did not reverse the anti-viral effect of IL-6. This is most
498 likely because IL-6 also activated STAT1 and STAT3 that lead to the induction of
499 STAT1-downstream anti-viral genes, IRF1, IRF7, and IRF9. Therefore the data suggest
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
504 that TMEV induced IL-6 production is under translational or inhibitory RNA control,
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
507 compared with B10.S macrophages. In one study K homology (KH)-type splicing
508 regulatory protein (KHSRP) was shown to target the AU-rich elements in the IL-6 mRNA
509 3' untranslated region which restricts its translation but does not lead to degradation of
510 mRNA (6). In another study microRNA-365 was shown to inhibit the translation of IL-6
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
513 could contribute to susceptibility versus resistance of macrophages to TMEV.

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

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 TMEV-susceptible 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 TMEV-induced 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 IFN-beta, 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

persistent TMEV infection of macrophage populations. Nevertheless, this study suggests therapeutic strategies that promote early IL-6 antiviral pathways could prevent chronic viral infections.

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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 μg/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 IFNβ 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 $p \leq 0.05$, ** $p \leq 0.01$.

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 $p \leq 0.05$.

758

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 x 10^6 macrophages
762 were untreated (control) or pretreated with U0126 (U) for 30 min before stimulation with
763 1 μg/ml LPS (A, B) or 50 μg/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 *t* test. * indicates
766 comparisons considered significantly different; $p \leq 0.05$.

767

768 **FIG 4** IL-12 and IFNβ enhance IL-6 expression and decrease TMEV replication during
769 TMEV infection of B10.S and SJL/J macrophages. 1 x 10^6macrophages from SJL/J and
770 B10.S mice were untreated (control) or treated with IL-12 p70 (1 ng/ml), IFNβ (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; $p \leq 0.05$.

776

777

778

779

780 **FIG 5** IL-12 and ERK-MAPK contribute to IL-6 expression during TMEV infection of
781 RAW264.7 macrophages. 1 x 10^6RAW264.7 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 x 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 $p \leq 0.05$, ** $p \leq 0.01$, n.d.=
787 not detectable.

788

789 **FIG 6** IL-6 constrains TMEV replication during infection of RAW264.7 macrophages. 1
790 x 10^6RAW264.7 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 $p \leq 0.05$, ** $p \leq 0.01$.

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 IFNβ with or without TMEV infection.
799 (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

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

819 Figure 1. Moore et al.

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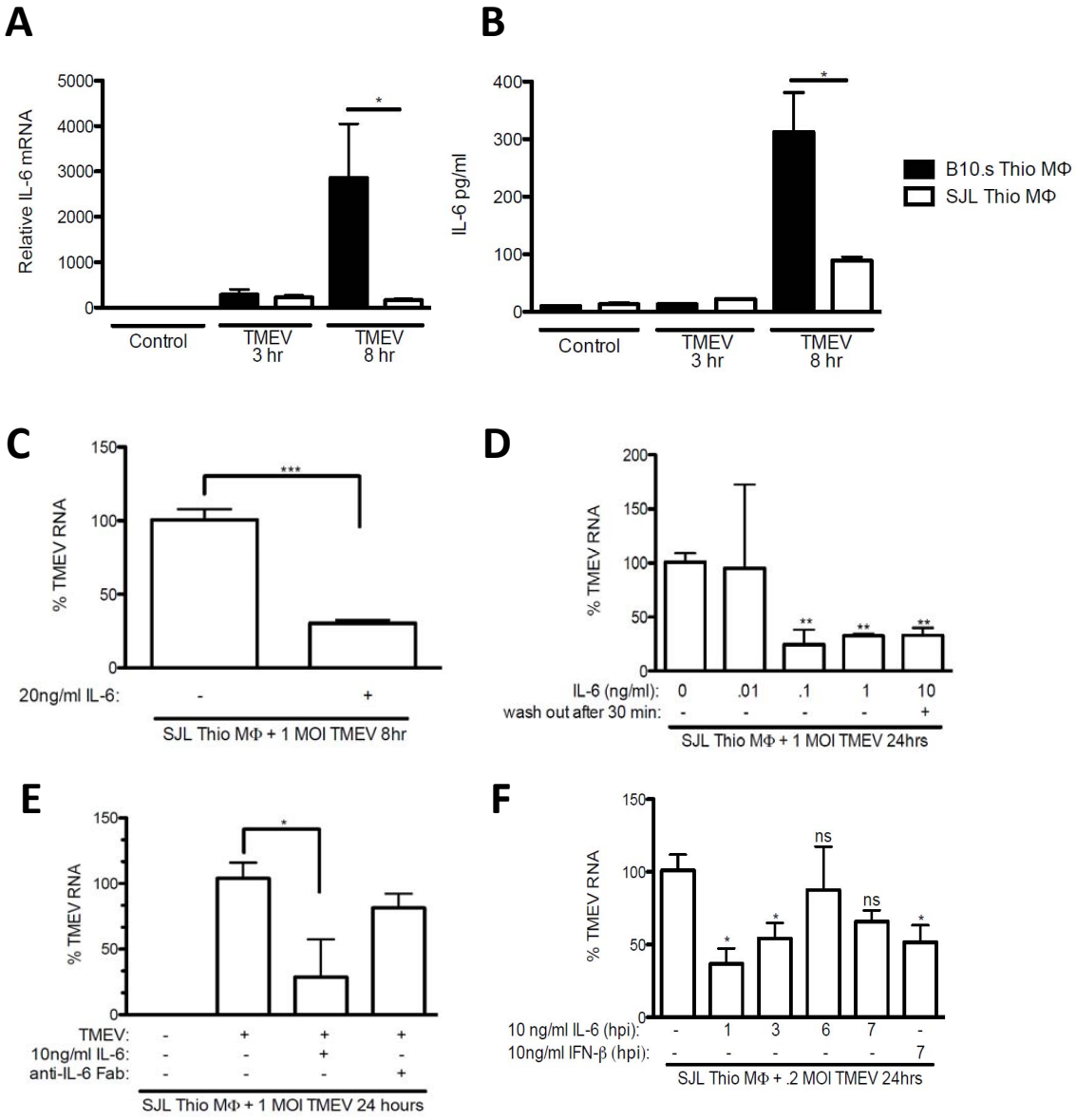
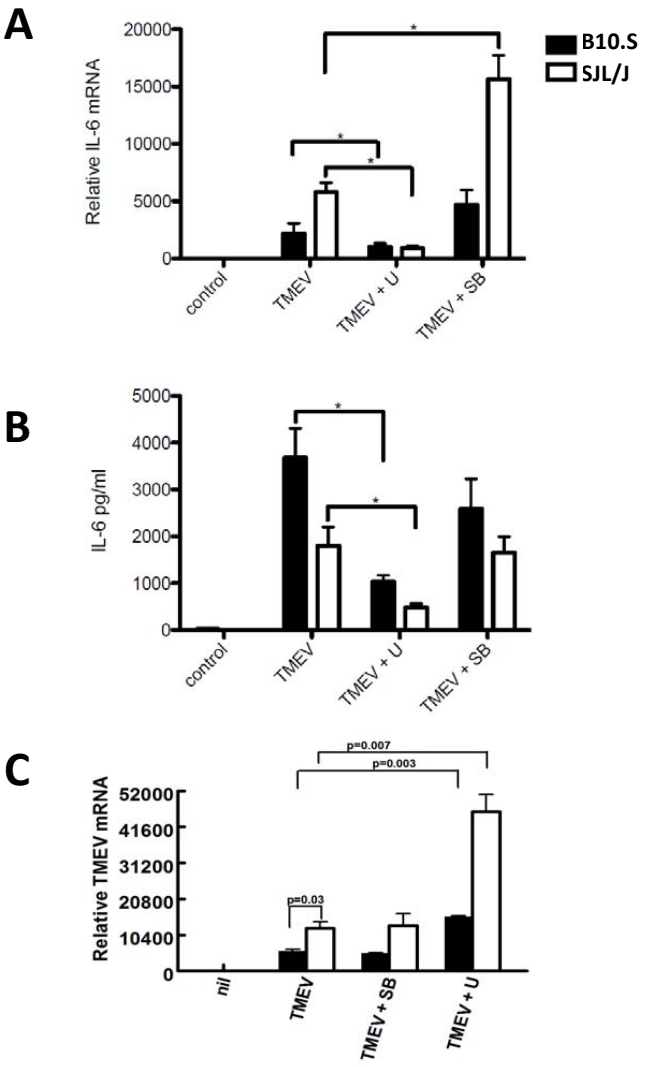
821



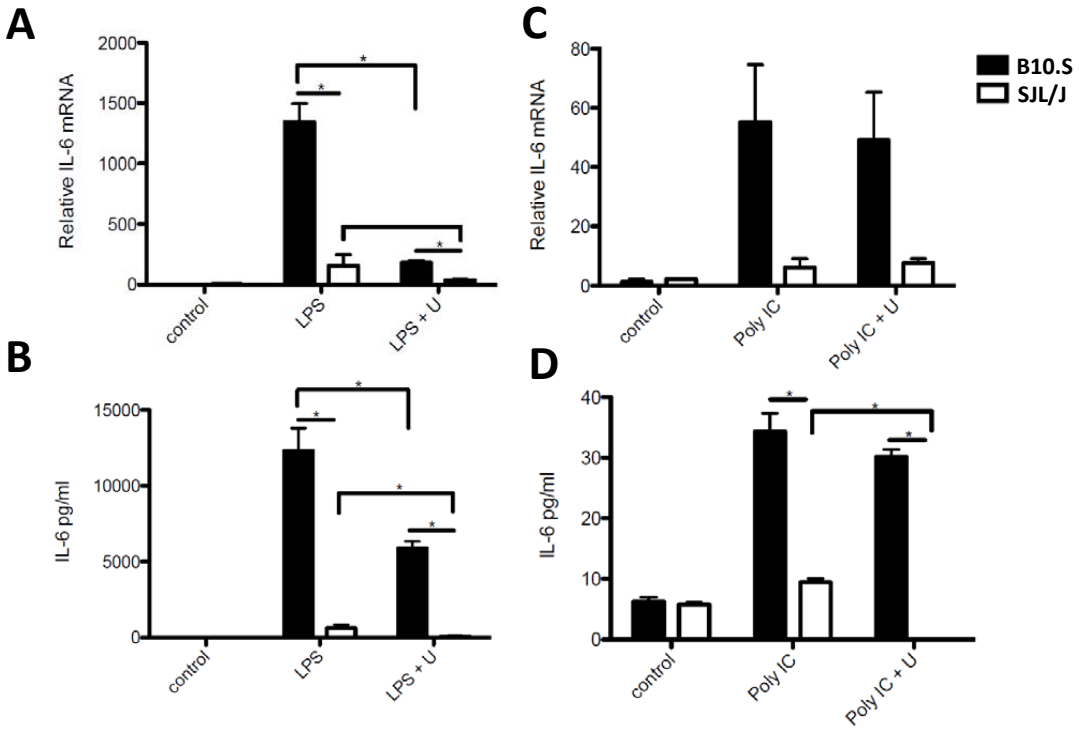
822 Figure 2. Moore et al.

823



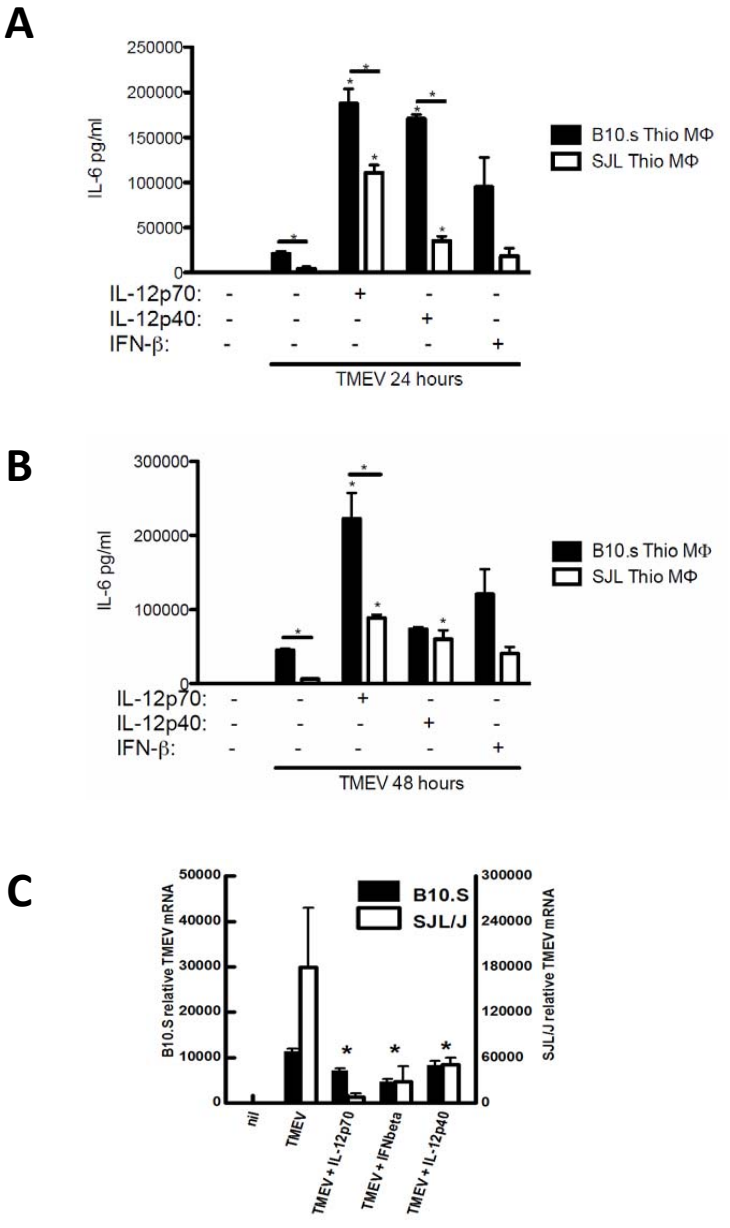
824 Figure 3. Moore et al.

825



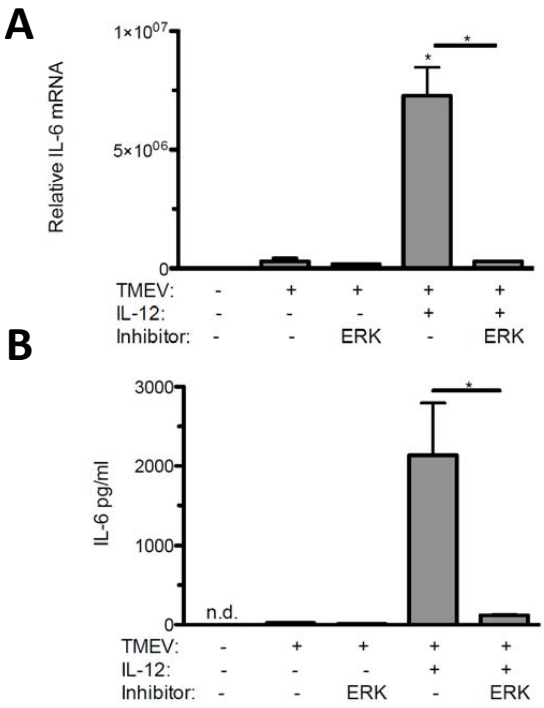
826 Figure 4. Moore et al.

827



828 Figure 5. Moore et al.

829



830 Figure 6. Moore et al.

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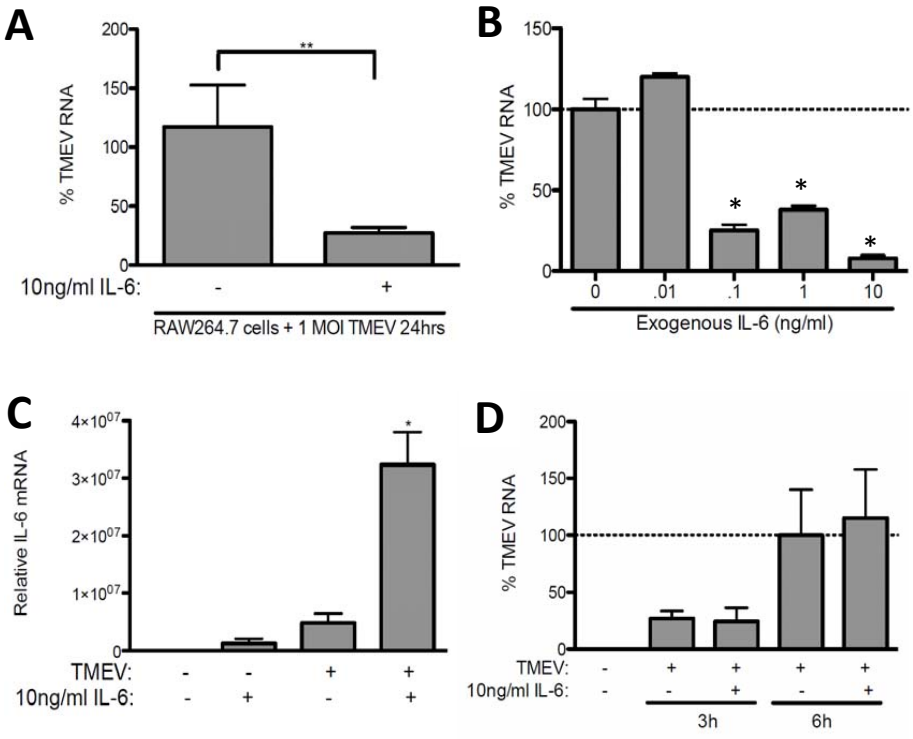

Figure 7. Moore et al.