Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 76 (2007) 159–167
Antiviral Research
www.elsevier.com/locate/antiviral

Expression of IL-27 p28 by Theiler's virus-infected macrophages depends on TLR3 and TLR7 activation of JNK-MAP-kinases

Lara Hause, Fahd M. Al-Salleeh, Thomas M. Petro *

Department of Oral Biology and the Nebraska Center for Virology, University of Nebraska Medical Center, Lincoln, NE 68583, USA

Received 22 February 2007; accepted 21 June 2007

Abstract

Theiler's murine encephalomyelitis virus (TMEV) causes a demyelinating disease (DD) due to infection of macrophages, stimulation of macrophage Toll-like receptor (TLR)3 and TLR7 pathways, activation of Mitogen-activated protein kinases (MAPK)s, and production of macrophages cytokines. Because expression of IL-27, a macrophage cytokine composed of p28 and EBI3 subunits, has been implicated in DD, we examined IL-27 subunit mRNA expression during TMEV infection of RAW264.7 cells, a macrophage cell line. TMEV infection of RAW264.7 cells did not affect cell viability, resulted in viral RNA replication, as well as p28 and EBI3 expression. Expression of p28 in TMEV-infected RAW264.7 cells depended on TLR3 and TLR7, as well as JNK but not p38 or ERK MAPKs. Since TMEV causes DD in SJL/J but not B10.S mice we determined the difference in expression of IL-27 subunit mRNA in SJL/J compared to B10.S macrophages. SJL/J macrophages expressed significantly more p28 mRNA after TMEV infection and after stimulation with TLR3 and TLR7 agonists compared with B10.S macrophages. Therefore, macrophages expression of IL-27 p28 mRNA in response to TMEV is due to activation of TLR3, TLR7, and JNK MAPKs pathways.

Keywords: IL-27; TMEV; JNK; TLR3; TLR7; Macrophages; RAW264.7 cells

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease (DD) in humans (Hafler, 2004) for which some evidence suggests that certain virus infections may be the cause (Alotaibi et al., 2004; Antony et al., 2004; Haahr et al., 2004; Lang et al., 2002; Levin et al., 2005; Sundstrom et al., 2004). Theiler's murine encephalomyelitis virus (TMEV), a picornavirus, infects cells of macrophage lineage and induces an autoimmune DD that resembles MS in the SJL/J but not the B10.S mouse strain. Most notably, TMEV infects macrophages, which infiltrate the brain, and microglial cells, which are resident macrophage-lineage cells in the brain (Olson et al., 2001). Both of these cell types are persistently infected with TMEV and produce nitric oxide which damages myelin (Pahan et al., 2001). However, subsequent to infection with TMEV, the antigen-presenting functions and cytokine expressions of microglial cells are enhanced (Olson et al., 2001). These TMEV-activated microglial cells process TMEV and myelin proteins, present TMEV and myelin epitopes, and secrete cytokines associated with development of CD4 T cell cytokine phenotypes of the adaptive immune response. Therefore, the cytokines produced during the innate anti-viral immune response to TMEV are an important consideration.

The infection of macrophages by viruses initiates an innate anti-viral immune response through Toll-like receptors (TLRs) (Steer et al., 2006) and mitogen-activated protein kinases (MAPKs) (Alexopoulou et al., 2001) resulting in production of cytokines (Petro, 2005a; Petro, 2005b). Among these cytokines, IFN-β, induces an anti-viral state and increases Natural killer (NK) cell cytotoxicity (Biron et al., 1999), while IL-12 induces NK cell proliferation (Biron et al., 1999; Nguyen et al., 2002), NK cell production of IFN-γ (Chan et al., 1991) and macrophage nitric oxide (Pahan et al., 2001), all of which have potent anti-viral properties. This innate immune response also influences the acquired anti-viral immune response when viral antigen is presented to CD4 T cells by macrophages (Hsieh et al., 1993; Schijns et al., 1995). These CD4 T cells in turn produce certain sets of cytokines which further contributes to anti-viral immunity. However, the innate macrophage anti-viral immune response could also be a factor in development of autoreactive

* Corresponding author at: Department of Oral Biology, University of Nebraska Medical Center, 40th and Holdrege Sts., Lincoln, NE 68583–0740, USA. Tel.: +1 402 472 1327; fax: +1 402 472 2551.
E-mail address: tpetro@unmc.edu (T.M. Petro).

0166-3542/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2007.06.013

CD4 effector T cells that promote DD (Charlton and Lafferty, 1995).

IL-27 is a newly identified member of the IL-12 family of macrophage cytokines that plays a role in the development of the CD4 T cell cytokine phenotype (Pflanz et al., 2002). It consists of a p28 subunit coupled to a subunit termed the Epstein Barr Virus (EBV)-induced gene 3 (EBI3). EBI3, which is related to IL-12/IL-23 p40 subunit, has been shown to be expressed in EBV-transformed B cells, tonsils, spleens, and placental trophoblasts (Devergne et al., 1997; Devergne et al., 1996). In addition, EBI3 can heterodimerize with the p35 subunit of IL-12. However, the function of EBI3/p35 is unknown. Nevertheless, the EBI3/p28 dimer, IL-27, promotes development and proliferation of Th1 CD4 T cells from naïve, but not memory, CD4 T cells. In another MS mouse model, EAE, neutralization of IL-27 p28 leads to decreased DD (Goldberg et al., 2004). However very recently, mice made deficient in the IL-27 receptor were more susceptible to development of EAE (Batten et al., 2006). Because TMEV infects macrophages it may stimulate production of IL-27. The exact mechanisms by which IL-27 subunits are induced from macrophages are unknown. We hypothesize that TLRs that activate p38, ERK, or JNK MAPKs are involved in TMEV induction of IL-27.

We have utilized the RAW264.7 cell line and peritoneal macrophages to examine the cell signaling pathways associated with expression of IL-12 and IL-23 in response to TMEV infection (Petro, 2005a; Petro, 2005b). We have shown that the expression of IL-23 p19 is dependent upon ERK MAPKs while expression of IL-23 p40 is dependent upon p38 MAPK. Thus IL-27 mRNA expression in TMEV-infected, RAW264.7 macrophage cell line, as well as SJL/J and B10.S primary macrophages was assessed utilizing real-time PCR. To determine which TLRs and MAPKs determine TMEV-induced IL-27 expression, RAW264.7 cells or peritoneal macrophages were stimulated with TLR3, 4, or 7 agonists. In addition, RAW264.7 cells were transfected with shRNA vectors specific for TLR3 and TLR7 or were pretreated with p38, ERK, or JNK MAPK inhibitors before TMEV infection. The results indicate that TMEV infection induced IL-27 p28 and EBI3 mRNA expression from RAW264.7 cells and induced significantly more IL-27 p28 in macrophages from SJL/J than B10.S mice. Expression of IL-27 p28 in RAW264.7 cells was dependent on activation of JNK-MAPKs and induced through TLR3 and TLR7 pathways.

2. Materials and methods

2.1. Experimental animals, cells, virus, and reagents

Female B10.S and SJL/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). RAW264.7 cells, a mouse macrophage cell line originally obtained from the American Type Culture Collection (Rockville, MD), were also grown in cell culture medium, seeded at 1.0×10^6 per well of a 6-well plate in cell culture medium and incubated at 37 °C. The DA strain of TMEV was obtained from Dr. Kristen Drescher, Department of Medical Microbiology and Immunology, Creighton University, Omaha, NE. TMEV was grown in BHK-21 cells to produce stocks with 1×10^7 PFU/ml. All experiments using animals were approved by the University of Nebraska Institutional Animal Care and Use Committee. Macrophages were obtained from the peritoneal cavity 3 days after intraperitoneal injection of 2 ml thioglycollate broth into SJL/J and B10.S mice. Peritoneal macrophages were incubated at 1×10^6 cells/2 ml of DMEM cell culture medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen), and 50 μg/ml gentamycin (Invitrogen). After 24 h, non-adherent cells were removed and 1 ml of cell culture medium added. TLRs stimulants were loxoribine (InVivoGen, San Diego, CA) an agonist of TLR-7, poly(IC) (InVivoGen) an agonist of TLR-3, or *E. coli* LPS 127:B8 (InVivoGen) an agonist of TLR-4.

2.2. Treatment and stimulation

Peritoneal macrophages or RAW264.7 cells seeded at 1×10^6 were untreated (control) or infected with 0.1, 0.5, 1.0, or 2.0×10^6 PFU of TMEV. Alternatively cells were untreated or pretreated for 30 min before infection with 1 μl SP 600125, an inhibitor of JNK MAPK (10 μM), and, 1 μl SB 203580 (10 μM), an inhibitor of p38 MAPK, 2 μl U0126 (20 μM), an inhibitor of ERK MAPK, or 1 μl DMSO carrier. Still further, cells were stimulated with 200 μM loxoribine, an agonist of TLR-7, 50 μg/ml poly(IC), an agonist of TLR-3, or 500 ng/ml *E. coli* LPS 127:B8 an agonist of TLR-4. RNA was collected at 3, 6, 9 or 24 h for real-time PCR. For western blot, cell extracts were collected at 30 min.

2.3. RNA interference

RAW264.7 cells were transfected with vectors which express shRNA specific for murine TLR3 (si-mTLR3) or TLR7 (si-mTLR7) (InvivoGen). The sequences of the siRNA that are generated from these vectors are: (only the sense strands are shown): si-mTLR3, 5′-GAGCATCAATCTAGGACTGAA-3′, and si-mTLR7, 5′-GATCTGCCATCCAGCTTACAT-3′. RAW264.7 cells were transfected using the Cell Line Nucleofector Kit specific for RAW264.7 cells (Amaxa Biosystems, Gaithersburg, MD). Transfected cells were seeded at 1×10^6 onto 6-well plates. Cells were stimulated as described above and after 24 h cell extracts were obtained for TLR3 and TLR7 real time-PCR.

2.4. Cell viability

RAW264.7 cell viability after TMEV infection was evaluated using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay of Promega (Madison, WI). Briefly, RAW264.7 cells were distributed to 96-well plates at 5000 cells per 100 μl in cell culture medium. PFU of TMEV ranging from 500 to 50,000 were added to respective wells and plates were incubated at 37 °C for 24 h. 20 μl of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/phenazine methosulfate (PMS) solution was added to each well to yield 317 μg/ml of MTS and 7.3 μg/ml PMS. Plates were incubated for 4 h at

37 °C in 5% CO_2. The number of infected cells at 24 h was determined by regression analysis of absorbencies at 490 nm of uninfected RAW264.7 cells ranging from 5×10^3 to 5×10^5 that were incubated with MTS/PMS.

2.5. Relative mRNA measurement by real time-PCR

RNA was extracted using the RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNAs were prepared by mixing 1 μg of RNA with 1 μM olidodeoxy-thymidine, 0.5 mM each of dATP, dGTP, dTTP, and dCTP, 20 U of RNAse inhibitor, and 525 U of MMLV reverse transcriptase (Invitrogen) in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$. After an incubation at 42 °C for 90 min followed by 94 °C for 5 min one fiftieth of the cDNA was mixed with primer pairs of genes of interest were: p28 sense primer 5′ AGCCTGTTGCTG CTACCCTTGC 3′, antisense primer 5′ GTGGACATAGCCCTGAACCTCA; EBI3 sense primer 5′ TCTTCCTGTCACTTGCCCTCTG 3′, antisense primer 5′ AGTTGGGAGCCTGGAGAGGAGT 3′; TMEV sense primer 5′ CTTCCCATTCTACTGCAATG 3′, antisense primer 5′ GTGTTCCTGGTTTACAGTAG 3′; GAPDH 5′sense primer 5′ TTGTCAGCAATGCATCCTGCAC 3′; antisense primer 5′ 5′ACAGCTTTCCAGAGGGGCCATC 3′. Real-time PCR reactions were run on an ABI Prism 7000 thermal cycler at 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s/60 °C for 30 s. Relative levels of mRNA for each factor were normalized to GAPDH determined by using the Ct value and the formula: $2^{\Delta\Delta}$Ct.

2.6. PAGE and Western immunoblot

Overnight RAW264.7 cells seeded at 1×10^6 were untreated or pretreated with SP 600125, SB203580 or U0126 30 min before infection with 1×10^6 PFU of TMEV. After 30 min, cells were disrupted with lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. 10 μg of protein were run on a 10% SDS, Tris–glycine–polyacrylamide gel, transferred to a PVDF membrane, which was then treated with LiCor blocking buffer. Membranes were then incubated in 1:1000 dilutions of mouse IgG specific for phospho-p44/42 (ERK), phospho-p38, rabbit IgG for p42/44 (ERK), mouse antibody to JNK, rabbit antibody to phospho-JNK, or mouse antibody to JNK (Cell Signaling Technology, Boston, MA). 1:1000 dilution of IRDye® 800CW goat anti-rabbit IgG (Rockland Immunochemicals Inc., Gilbertsville, PA) combined with Alexa Fluor® 680 goat anti-mouse IgG (Molecular Probes/Invitrogen). The membrane was washed three times and then scanned with a LICOR Odyssey® Infrared Imaging System.

2.7. Statistical analysis

Data were analyzed by the Student *t*-test to determine the significance of differences between the mean values. *P*-values of less than 0.05 were considered to be significant.

3. Results

3.1. RAW264.7 infection with TMEV

We have utilized TMEV infection of RAW264.7 cells in order to gain an understanding of signaling mechanism for IL-12 and IL-23 expression during viral infection (Petro, 2005b). Therefore, our aim was to again utilize RAW264.7 cells to gain an understanding of signaling mechanisms in IL-27 p28 and EBI3 expression. However, we thought it important to first optimize the conditions for infection of RAW264.7 cells. To establish the temporal pattern in the TMEV infection of RAW264.7 cells we measured relative TMEV RNA levels in RAW264.7 cells by real-time PCR at 3, 6, 9, and 24 h after infection with an MOI of 1. TMEV RNA in RAW264.7 cells steadily increased for up to 9 h after infection after which it decreased at 24 h (Fig. 1A). Therefore, TMEV infection of RAW264.7 cells hits the highest point at 9 h after infection. We also assessed various MOIs of TMEV (0.1, 0.5, 1.0, and 2.0) to establish which MOI leads to the highest expression of TMEV RNA in RAW264.7 cells. RAW264.7 cells exhibited a steady increase in TMEV RNA that was proportional to the MOI (Fig. 1B). It is possible that TMEV infection of macrophages could modulate cytokine production by affecting macrophage viability. To establish the impact that TMEV infection has on RAW264.7 cell viability we infected 5×10^3 RAW264.7 cells with various doses of TMEV ranging from 0 to 5×10^3 PFU and measured cell viability at 24 h using an MTS assay, which depends upon the conversion of tetrazolium to a soluble colorimetric formazan product by viable cells. TMEV infection of RAW264.7 cells had no detectable effect upon cell viability for up to 24 h after infection (Fig. 1C). Therefore, any effect that TMEV infection has on cytokine expression is not due to effects upon RAW264.7 cell viability. Overall, these results show that TMEV infection of RAW264.7 cells reaches its highest point at 9 h with an MOI of 2.0.

3.2. TMEV induces expression of IL-27 p28 and EBI3 mRNA

To establish that RAW264.7 cells express IL-27 subunits in response to TMEV infection and to establish the conditions for expression, RAW264.7 cells, seeded at 1×10^6, were incubated with 1×10^6 TMEV PFU, and relative p28 and EBI3 mRNA expression was measured by real-time PCR at 3, 6, 9, and 24 h after infection. TMEV-induced significant expression of p28 mRNA from RAW264.7 cell at 6, 9, and 24 h after infection with peak expression of mRNA at 6 h (Fig. 2A). Likewise, TMEV-induced significant EBI3 mRNA at 6 h after infection in RAW264.7 cells (Fig. 2B). However, EBI3 mRNA at 3, 9, and 24 h after infection was not significantly different than background levels of expression. Therefore, TMEV induces both IL-27 subunits in RAW264.7 cells at 6 h after infection. To establish the optimal MOI for simultaneous induction of both p28 and EBI3 mRNA, RAW264.7 cells were infected for 24 h with various TMEV MOI (0.1, 0.5, 1.0, and 2.0). Expression of p28 mRNA was proportional to the increase in TMEV MOI with the highest level of expression of p28 at an MOI of 2.0 (Fig. 2C).

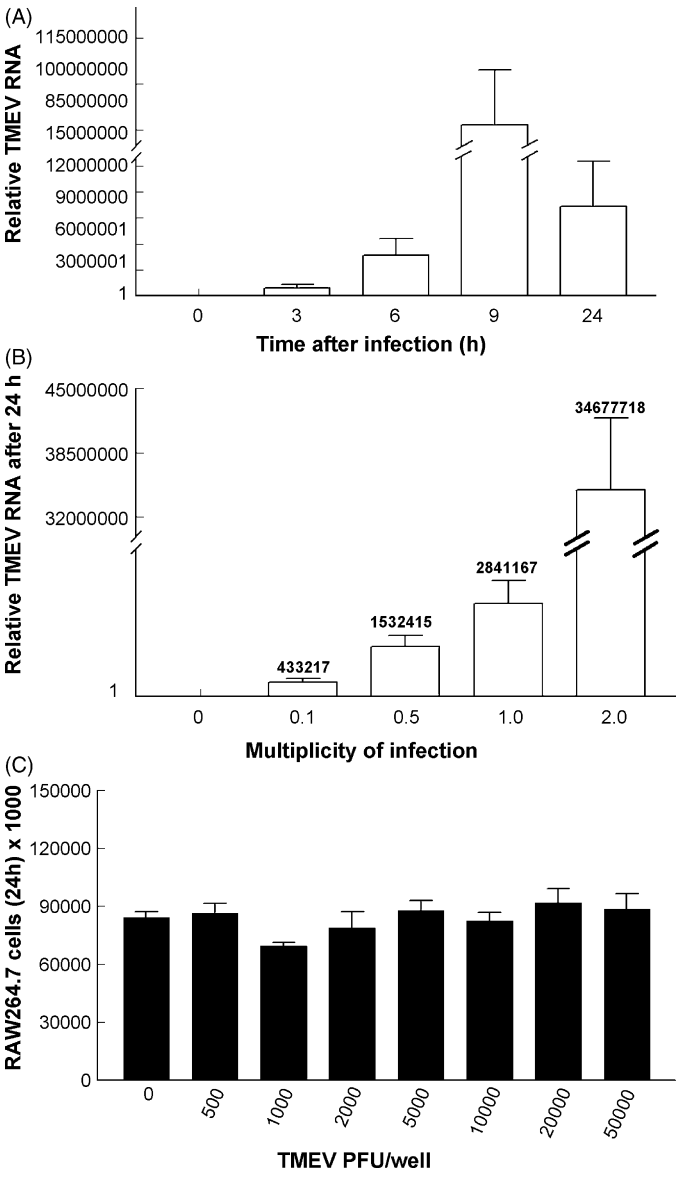

Fig. 1. TMEV RNA expression in TMEV-infected RAW264.7 cells. TMEV RNA in (A) 1×10^6 RAW264.7 cells at 3, 6, 12, and 24 h after infection with 1×10^6 TMEV; (B) 1×10^6 RAW264.7 cells at 24 h after infection with 0.1, 0.5, 1.0, or 2.0 TMEV MOI. RNA was reverse transcribed and relative levels of TMEV cDNA were measured by real-time PCR normalized to GAPDH cDNA. Viability 5×10^3 RAW264.7 cells (C) at 24 h after infection with various doses of TMEV ranging from 0 to 5×10^4 PFU as measured with an MTS assay. Data are means of four samples each of a representative experiment.

In contrast, significant TMEV-induced EBI3 mRNA was only achieved at an MOI of 2.0 (Fig. 2D). Therefore, RAW264.7 cells will express both subunits of IL-27 in response to TMEV at 2.0 MOI. However, at lower MOIs p28 is induced without significant induction of EBI3.

3.3. TLR3 and TLR7 signaling induces expression of the IL-27 p28 subunit

RAW264.7 cells express most TLRs including TLR3, which recognizes viral dsRNA, and TLR7, which recognizes viral ssRNA (Applequist et al., 2002). Because TMEV is a picornavirus it is expected to stimulate RAW264.7 cells through TLR7 and TLR3. To establish that these TLR pathways induces IL-27 p28 and EBI3 mRNA RAW264.7 cells were stimulated for 24 h with a TLR3 agonist (poly(IC)), a TLR7 agonist (loxoribine), or as a positive control, a TLR4 agonist (LPS). Poly(IC) but not LPS or loxoribine induced p28 mRNA expression in RAW264.7 cells significantly above background (Fig. 3A). In contrast, none of the TLR agonists induced significant EBI3 mRNA expression in RAW264.7 cells, although EBI3 expression tended to be higher in RAW264.7 cells stimulated with loxoribine (Fig. 3B).

Short hairpin RNA (shRNA) vectors which produce short inhibitory (si)RNAs decrease expression of target proteins (Elbashir et al., 2001; Amarzguioui et al., 2005), including TLRs (Chen et al., 2006). To determine the significance of TLR3 and TLR7 to IL-27 p28 expression by RAW264.7 cells, we transfected RAW264.7 cells with expression vectors encoding shRNA specific for TLR3 or TLR7 before infection with 2.0 MOI of TMEV. The TLR3 and TLR7 shRNA expression vectors decreased expression of TLR3 and TLR7 protein, respectively, in RAW264.7 cells such that TLR3 and TLR7 were not detectable by Western immunoblot (data not shown). After TMEV infection, RAW264.7 cells transfected with TLR3 or TLR7 expression vectors exhibited a significant reduction in TMEV-induced p28 mRNA compared with RAW264.7 cells transfected with empty plasmid vectors (Fig. 3C). Therefore, TMEV stimulates expression of IL-27 p28 expression through TLR3 and TLR7.

3.4. JNK MAPKs are required for expression of IL-27 p28 mRNA

Stimulation through TLR3 and TLR7 pathways leads to activation of p38, ERK, and JNK MAPKs, which are also responsible for cytokine expression by macrophages (Jiang et al., 2003; Jones et al., 2001). To determine which MAPK pathway(s) are responsible for TMEV-induced IL-27 p28 and EBI3, RAW264.7 cells were pretreated with inhibitors of p38, ERK, or JNK MAPK pathways before infection with TMEV as we have done previously (Petro, 2005a; Petro, 2005b). SB203580 inhibits activation of components downstream of the p38 pathway such as ATF-2 (Petro, 2005b) while U0126 and SP 600125 inhibit phosphorylation of the ERK and JNK MAPKs, respectively (Fig. 4A). TMEV-induced p28 mRNA was significantly reduced by the JNK MAPK inhibitor, SP 600125, but was significantly increased by the ERK MAPK inhibitor, U0126, and was not affected by the p38, inhibitor, SB203580 (Fig. 4B). In contrast, expression of EBI3 mRNA was not affected by any of the MAPK inhibitors (Fig. 4C). Therefore, TMEV-induced IL-27 p28 mRNA depends on activation of the JNK MAPKs but controlled by activation of the ERK MAPKs. It is possible that the SP600125 could have modulated TMEV-induced p28 mRNA because it modulated virus replication. Therefore, we were curious to see if the MAPK inhibitors had any affect on TMEV RNA levels. Pretreatment of RAW264.7 cells with SB203580 or U0126 had no effect on TMEV RNA levels. Interestingly, pretreatment with SP 600125 significantly increased TMEV RNA in

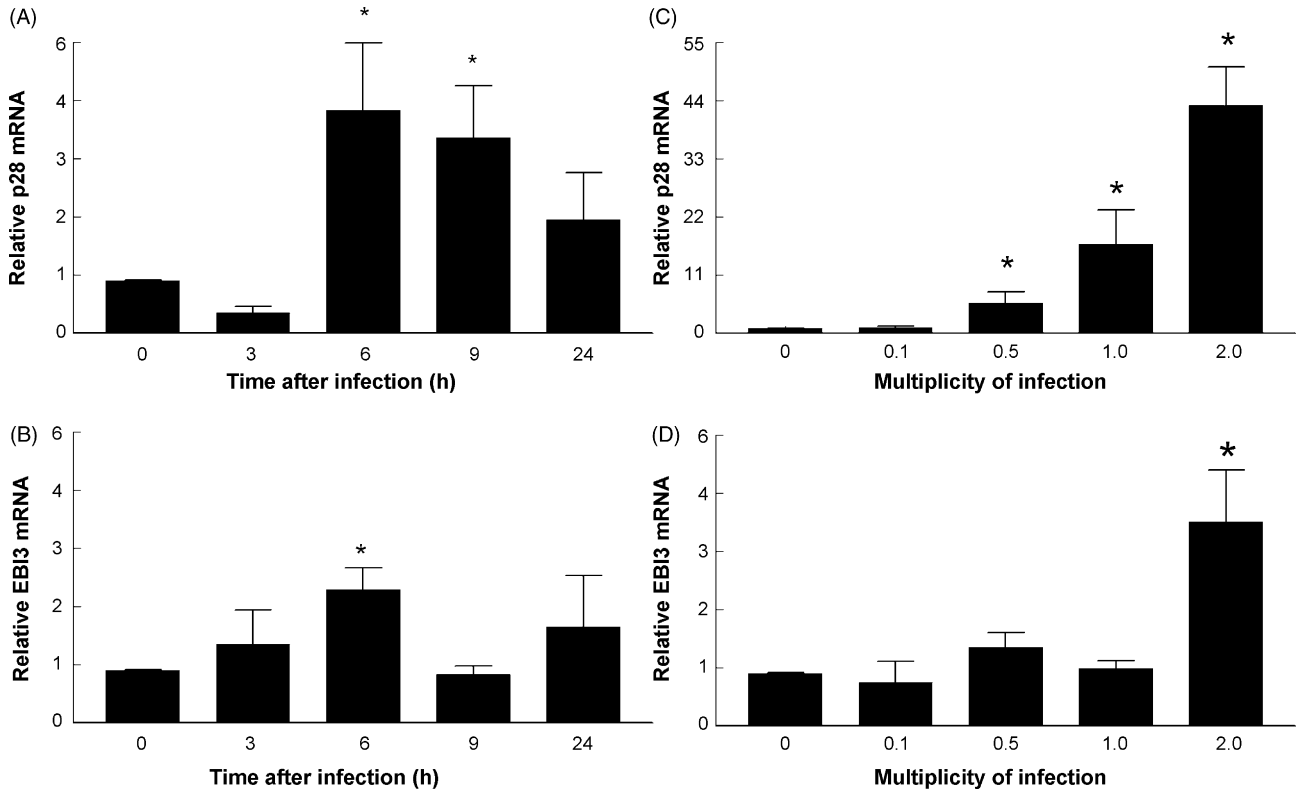

Fig. 2. IL-27 subunit expression in TMEV-infected RAW264.7 cell. IL-27 p28 (A and C) and EBI3 (B and D) mRNA expression in 1×10^6 RAW264.7 cells at 3, 6, 12, and 24 h after infection with 1×10^6 TMEV (A and B) or 24 h after infection with infection with 0.1, 0.5, 1.0, or 2.0 TMEV MOI (C and D). mRNA was reverse transcribed and relative levels of p28 and EBI3 cDNA were measured by real-time PCR normalized to GAPDH cDNA. Data are means of four samples each of a representative experiment evaluated by the Student *t*-test; means which were considered significantly different than means from unstimulated cells are indicated by (*).

RAW264.7 cells (Fig. 5). Therefore, JNK MAPKs or IL-27 may control expression of TMEV RNA in infected macrophages.

3.5. IL-27 p28 and EBI3 mRNA expression is greater in SJL/J macrophages than in B10.S macrophages

TMEV infects SJL/J macrophages to a greater extent than B10.S macrophages and induces a differential expression of IL-12 p25 and p40 subunits (Petro, 2005a). To determine if TMEV induces a similar differential expression IL-27 p28 and EBI3 mRNA, SJL/J and B10.S macrophages were infected with TMEV for 24 h and relative cytokine mRNA expression was measured by real-time PCR. SJL/J macrophages expressed significantly more p28 mRNA in response to TMEV infection than B10.S macrophages (Fig. 6A). However, expression of EBI3 mRNA by SJL/J macrophages in response to TMEV, while tending to be higher, was not significantly different than B10.S macrophages (Fig. 6B).

The differential expression of IL-12 p35 and p40 by SJL/J and B10.S macrophages in response to TMEV infection was reflected in the response of these macrophages to TLR3 and TLR7 agonists (Petro, 2005a). To determine if stimulation through TLR3 or TLR7 pathways could also differentially induce IL-27 p28 and EBI3 mRNA, SJL/J and B10.S macrophages were stimulated with poly(IC), loxoribine and LPS. SJL/J macrophages expressed p28 mRNA in response to poly(IC), loxoribine, and LPS to a significantly greater extent than B10.S macrophages (Fig. 6A). Similarly, SJL/J macrophages expressed significantly more EBI3 mRNA in response to poly(IC) and LPS compared with B10.S macrophages. Interestingly, loxoribine did not significantly stimulate EBI3 mRNA from macrophages of either strain (Fig. 6B).

4. Discussion

The results of the present investigation clearly show that the RAW264.7 is an ideal macrophage cell line to examine signaling mechanisms essential for virus-induced expression of IL-27 subunits, p28 and EBI3 from macrophages. The RAW264.7 macrophage cell line has been useful for examining virus-activated signaling pathways which stimulate expression of IL-12 family of proteins (Petro, 2005b). Therefore, we utilized TMEV infection of RAW264.7 cells in order to gain an understanding of signaling mechanisms for TMEV induction of IL-27. We show herein that RAW264.7 cells simultaneously express p28 and EBI3 mRNA 6 h after infection with TMEV. This is significant because IL-27 stimulates naïve T cells, but not memory T cells, during the early adaptive immune response (Pflanz et al., 2002). Therefore, macrophages infected with TMEV will likely stimulate naïve T cells during this short time-frame. However, p28 and EBI3 mRNAs were not expressed simultaneously

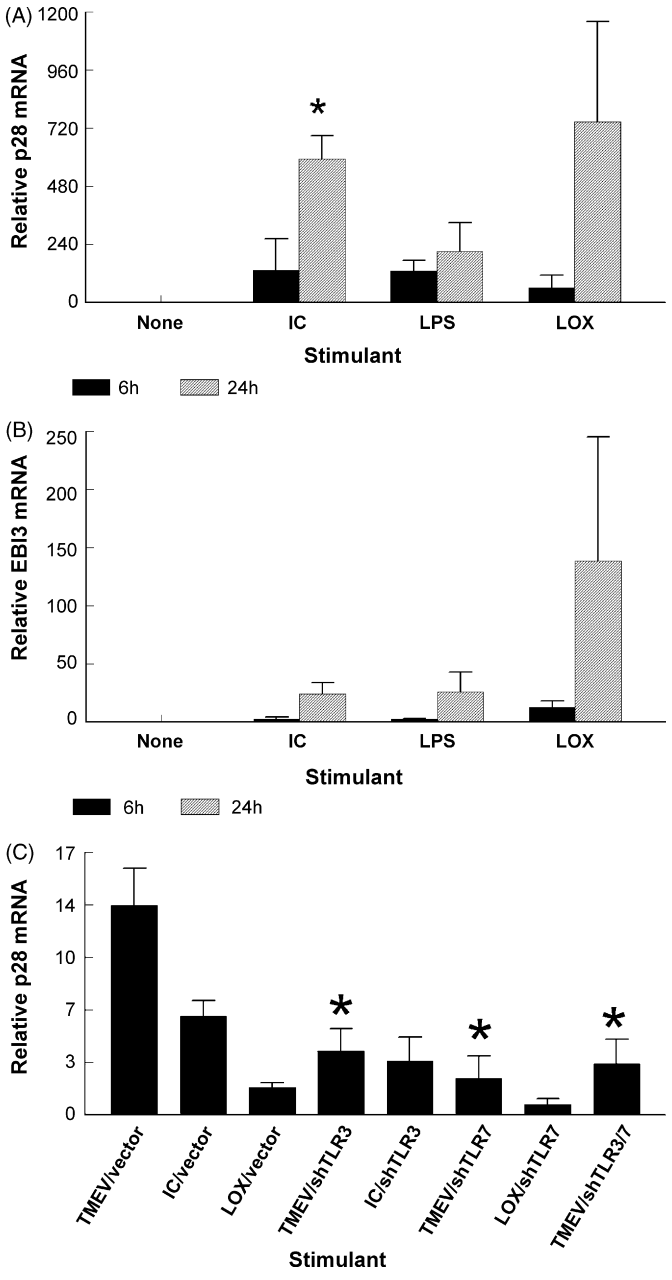

Fig. 3. IL-27 subunit expression in RAW264.7 cells stimulated with poly(IC), LPS, or loxoribine. IL-27 p28 (A) and EBI3 (B) mRNA expression in 1×10^6 RAW264.7 cells 24 h after stimulation with 200 μM loxoribine (LOX), 50 μg/ml poly(IC), or 500 ng/ml *E. coli* LPS 127:B8. (C) p28 mRNA expression in 1×10^6 RAW264.7 cells that were transfected with empty vector, shTLR-3, shTLR-7, or shTLR-3 plus shTLR-7. Following transfection cells were infected with 1×10^6 PFU of TMEV for 24 h. Bar graphs represent means ± S.E. of p28 or EBI3 cDNA relative to GAPDH cDNA of 2 independent experiments with 5 samples each. (*) Indicates that the mean is significantly different than controls at $\alpha = 0.05$ confidence level.

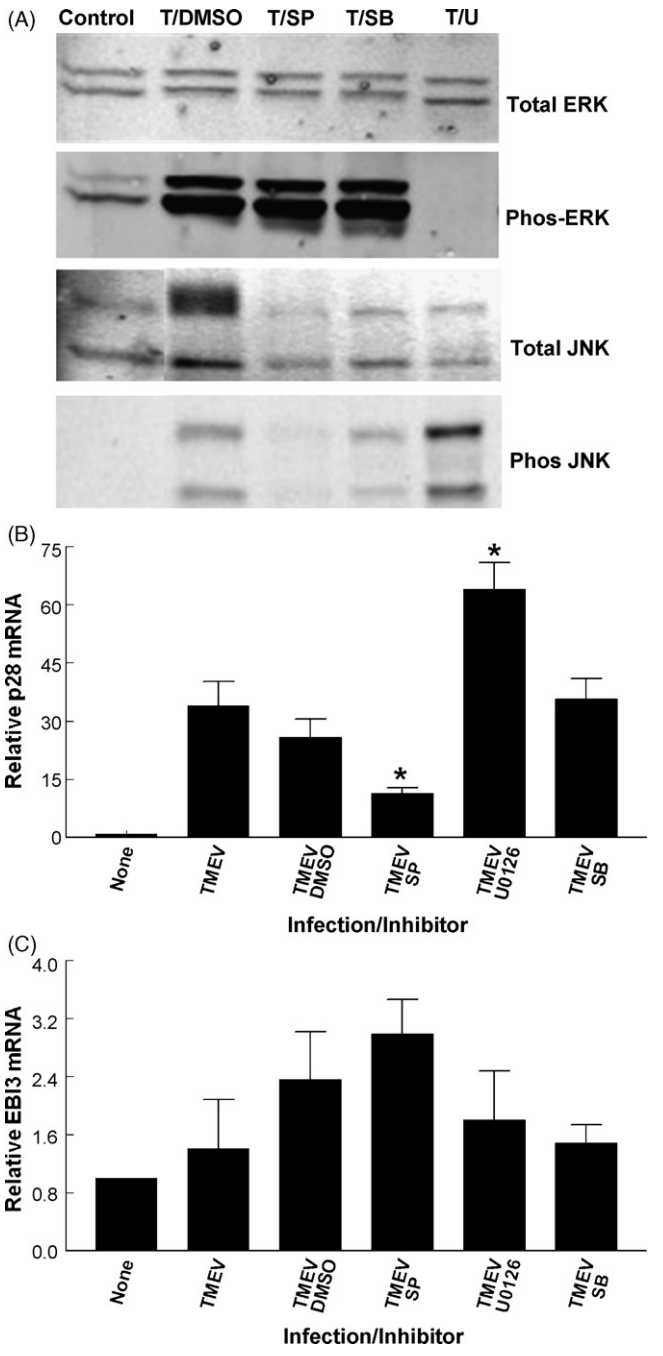

Fig. 4. MAPK Western blots (A) or IL-27 subunit expression (B and C) in TMEV-infected RAW264.7 cell pretreated with p38, ERK, and JNK MAPK inhibitors. Representative Western immunoblots (A) of phosphorylated-ERK(phos-ERK) or total ERK and phosphorylated-JNK (phos-JNK) or total JNK MAPKs in 10 μg of cell extract from RAW264.7 cells pretreated with 1 μl DMSO carrier, SP 600125 (SP; 10 μM), SB 203580 (SB; 10 μM), or U0126 (U; 20 μM) before infection with 1×10^6 TMEV. IL-27 p28 (B) and EBI3 (C) mRNA expression in 1×10^6 RAW264.7 cells pretreated with SP, U, or SB 30 min before infection with TMEV. After 6 h of infection, mRNA was reverse transcribed and relative levels of p28 and EBI3 cDNA were measured by real-time PCR normalized to GAPDH mRNA. Data are means of four samples each of a representative experiment evaluated by the Student *t*-test; means which were considered significantly different than means from unstimulated cells are indicated by (*).

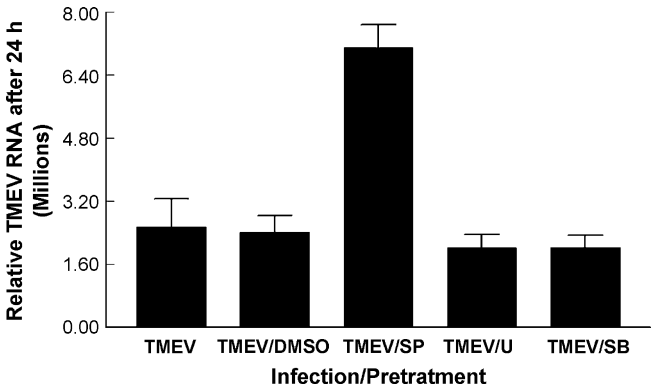

Fig. 5. TMEV RNA expression in TMEV-infected RAW264.7 cells pretreated with p38, ERK, and JNK MAPK inhibitors. TMEV RNA at 24 h in 1×10^6 RAW264.7 cells pretreated with SP 600125 (SP; 10 μM), SB 203580 (SB; 10 μM), U0126 (U; 20 μM), or 1 μl DMSO carrier before infection with 1×10^6 TMEV. After 6 h, mRNA was reverse transcribed and relative levels of TMEV cDNA were measured by real-time PCR normalized to levels of GAPDH mRNA. Data are means of five samples each of a representative experiment.

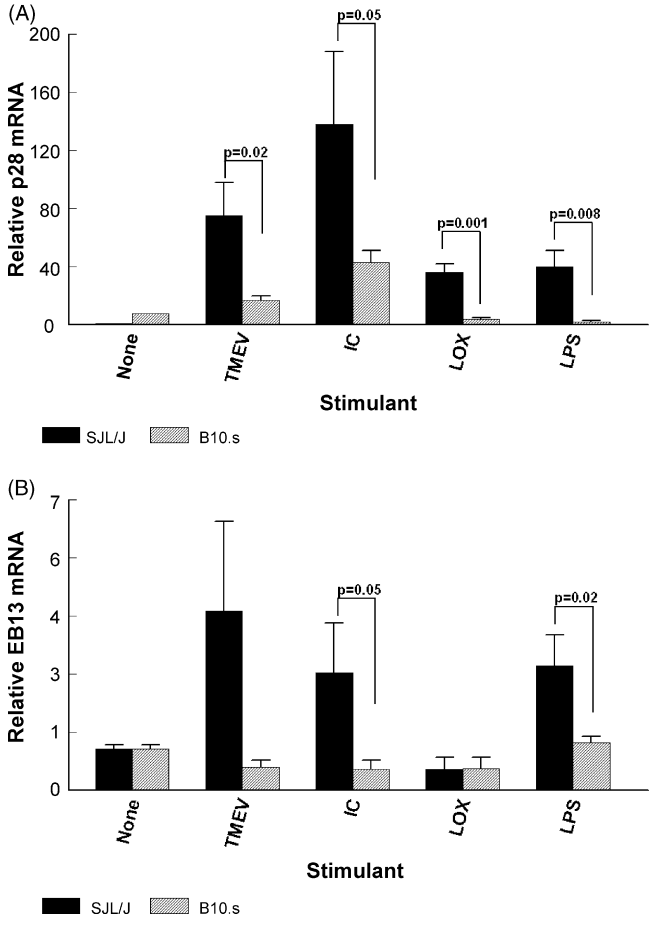

Fig. 6. IL-27 subunit mRNA expression in TMEV-infected macrophages from SJL/J and B10.S mice. IL-27 p28 (A) and EBI3 (B) mRNA expression in SJL/J and B10.S macrophages 24 h after infection with TMEV or stimulation with poly(IC), loxoribine (LOX), or LPS. 1×10^6 peritoneal macrophages were infected with 1×10^6 PFU of TMEV or stimulated with 50 μg/ml poly(IC), 200 μM LOX, or 500 ng/ml LPS for 24 h. mRNA was reverse transcribed and relative levels of p28 and EBI3 cDNA were measured by real-time PCR normalized to GAPDH cDNA. Data are means of four samples each of a representative experiment evaluated by the Student *t*-test; comparisons which were considered significantly different are indicated by brackets.

at all times after infection. The p28 mRNA was expressed for up to 24 h after TMEV infection in RAW264.7 cells. EBI3 mRNA was significantly induced only at 6 h after TMEV infection. Therefore, both subunits of IL-27 are not always expressed concurrently within RAW264.7 cells. Since, p28 is not efficiently secreted without EBI3 it is not likely that bioactive IL-27 is secreted beyond 6 h after TMEV infection. This discord could be due to the fact that p28 has a function by itself or has an alternate molecular partner besides EBI3, as do other IL-12 cytokine family members. The IL-12 p40 subunit combines with p35 to form IL-12 and also combines with p19 to form IL-23 (Oppmann et al., 2000). Likewise, IL-12 p35, which combines with p40, also combines with EBI3 (Devergne et al., 1997; Devergne et al., 1996). To date an alternate partner for p28 has not been identified, but an additional partner for p28 to form another IL-12 cytokine family member is still possible.

These results are significant because TMEV infection of macrophages has been associated with MS-like DD in mice (Lipton et al., 2005; Lipton et al., 1995). A previous study showed that neutralization of IL-27 with antibody to p28 leads to a reduction in the severity of DD in MS-like EAE (Goldberg et al., 2004). The prospect that viruses, which induce MS-like disease in mice, also induce subunits of IL-27 point out that the signaling pathways by which viruses such as TMEV induce expression of IL-27 subunits in macrophages needs to be determined. Therefore, further studies are required to determine if TMEV-induced IL-27 contributes to the development of MS-like disease in mice MS in humans.

At the onset of macrophage interactions with viruses, TLR pathways are critical to induction of cytokine expression. However, in addition to macrophages, astrocytes and microglial cells are also infected with TMEV (Lipton et al., 2005; Lipton et al., 1995) and these cells also express most TLRs (Olson and Miller, 2004) (Bsibsi et al., 2002; So et al., 2006). Therefore, these cells may also express IL-27. Herein we show that the TLR3 agonist, poly(IC) induces significant expression of IL-27 p28 mRNA but not EBI3 mRNA expression from RAW264.7 cells. While the TLR7 agonist, loxoribine, tended to induce p28 expression it was not significantly higher than background. However, a reduction in expression of TLR3 or TLR7 with shRNA vectors reduced TMEV-induced p28 mRNA expression. The discrepancy may stem from the fact that TMEV infection of RAW264.7 cells increases expression of TLR3 and TLR7 significantly above that found on uninfected RAW264.7 cells (data not shown) and a critical level of TLR7 expression is not achieved until after TMEV infection has begun. In any case, TLR3 and TLR7 are essential for expression of IL-27 p28 in response to TMEV.

Unlike other TLRs, TLR3 and TLR7 are endocytic membrane receptors for dsRNA and ssRNA, respectively (Barton et al., 2006; Hemmi et al., 2002; Takeuchi et al., 2004). In addition to IL-27, the TLR3 pathway is also responsible for induction of IFN-β and other cytokines from cells infected with TMEV (Kwon et al., 2004; So et al., 2006). Therefore, mechanisms to decrease the activity of the TLR3 or TLR7 pathways may be useful in treatment of MS-like disease. It should be noted that TLR8, which has significant homology to TLR7, also binds to ssRNA and mice express TLR8 (Heil, 2004). However,

RAW264.7 cells do not express TLR8 (data not shown). Interestingly, initial reports indicated that murine TLR8 is not responsive to the same agonists as TLR7 but does respond to TLR8-specific agonists in the presence of oligodeoxynucleotides (Gorden et al., 2006). Therefore future studies should consider whether TMEV stimulates macrophage cytokine production through TLR8.

However, stimulation of the TLR3 (Jiang et al., 2003) and TLR7 (Heil et al., 2003) pathways activate p38, ERK, or JNK MAPKs (Dong et al., 2002). We show in this report utilizing RAW264.7 cells that in addition to TLR3 and 7, TMEV induces expression of IL-27 p28 through MAPK pathways. Specifically, expression of p28 required activation of the JNK MAPKs, but not p38 or ERK MAPKs. Several reports have shown that activation of the TLR3 pathway leads to activation of JNK MAPKs (Shim et al., 2005; Steer et al., 2006; Stewart et al., 2006) through TAK1 (Shim et al., 2005) and IRF-8 (Zhao et al., 2006). Therefore, it is now important to determine if macrophages infected with TMEV exhibit activated TAK1 or IRF-8. In contrast to JNK MAPKs, activation of ERK MAPKs appears to decrease expression of p28 since addition of the ERK MAPK inhibitor, U0126, increased expression of p28. Therefore, if IL-27 contributes to DD then treatment with the JNK MAPK inhibitor, SP 600125, may be useful. However, it should be noted that, while inhibition of JNK activation resulted in a decrease in p28 expression it also resulted in a significant increase in viral RNA replication in RAW264.7 cells. It could be that IL-27 is involved in innate anti-viral immunity of macrophages. Alternatively, JNK-MAPKs may be involved directly in controlling viral replication. Other reports have found JNK MAPKs to be involved in virus replication. Inhibition of JNK-MAPKs reduces Rotavirus, Herpes simplex 1 virus, and cytomegalovirus replication (Holloway and Coulson, 2006; McLean and Bachenheimer, 1999; Rahaus et al., 2004). Therefore, the mechanism by which JNK MAPKs are involved in TMEV replication must be determined.

We previously showed that expression of the p40 subunit of IL-12/IL-23 is significantly greater in TMEV-infected SJL/J compared with B10.S macrophages (Dahlberg et al., 2006; Petro, 2005a). In contrast, our previous report showed that expression of the p35 subunit of IL-12 by SJL/J macrophages in response to TMEV infection is significantly less than B10.S macrophages(Petro, 2005a). The results of the present investigation similarly show that macrophages from SJL/J mice, which are more susceptible to TMEV-DD, express significantly greater levels of the IL-27 subunits p28 and EBI3 than macrophages from B10.S mice, which are resistant to TMEV-DD (Lipton et al., 2005; Lipton et al., 1995; Monteyne et al., 1999). Our data suggest that future studies are required to determine if IL-27 production from TMEV-infected SJL/J macrophages contributes to TMEV-DD. In addition, TLR3 and TLR7 agonists induced greater expression of p28 in SJL/J macrophages compared with B10.S macrophages. These results suggest that the TLR3 and TLR7 pathways are more active in SJL/J macrophages compared with B10.S macrophages. Indeed we have shown that TLR3 and TLR7 mRNA expression in SJL/J macrophages is greater than B10.S macrophages (Petro, 2005a). Interestingly, EBI3 was not induced in B10.S macrophages by any of the TLR agonists, while only polyIC induced EBI3 in SJL/J macrophages and p28 in B10.S macrophages. Therefore, the pathways for EBI3 expression need to be elucidated. In summary our results suggest that the in vivo mechanism by which IL-27 is expressed in MS-like disease in mouse models is an interesting consideration for future studies. The results herein point to pathways involved in IL-27 subunit expression in response to TMEV infection of macrophages, namely TLR3/TLR7 and JNK-MAPKs.

Acknowledgements

This work was support by grants RG3381-A2 from the National Multiple Sclerosis Society and grant P20 RR15635 from the US NIH COBRE program of the National Center for Research Resources.

References

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-[kappa]B by Toll-like receptor 3. Nature 413, 732–738.

Alotaibi, S., Kennedy, J., Tellier, R., Stephens, D., Banwell, B., 2004. Epstein-Barr virus in pediatric multiple sclerosis. JAMA 291 (15), 1875–1879.

Amarzguioui, M., Rossi, J.J., Kim, D., 2005. Approaches for chemically synthesized siRNA and vector-mediated RNAi. FEBS Lett. 579 (26), 5974–5981.

Antony, J.M., van Marle, G., Opii, W., Butterfield, D.A., Mallet, F., Yong, V.W., Wallace, J.L., Deacon, R.M., Warren, K., Power, C., 2004. Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. Nat. Neurosci. 7 (10), 1088–1095.

Applequist, S.E., Wallin, R.P., Ljunggren, H.G., 2002. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. Int. Immunol. 14 (9), 1065–1074.

Barton, G.M., Kagan, J.C., Medzhitov, R., 2006. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat. Immunol. 7 (1), 49–56.

Batten, M., Li, J., Yi, S., Kljavin, N.M., Danilenko, D.M., Lucas, S., Lee, J., de Sauvage, F.J., Ghilardi, N., 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. Nat. Immunol. 7 (9), 929–936.

Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P., Salazar-Mather, T.P., 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol. 17, 189–220.

Bsibsi, M., Ravid, R., Gveric, D., van Noort, J.M., 2002. Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol. Exp. Neurol. 61 (11), 1013–1021.

Chan, S.H., Perussia, B., Gupta, J.W., Kobayashi, M., Pospisil, M., Young, H.A., Wolf, S.F., Young, D., Clark, S.C., Trinchieri, G., 1991. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J. Exp. Med. 173 (4), 869–879.

Charlton, B., Lafferty, K.J., 1995. The Th1/Th2 balance in autoimmunity. Curr. Opin. Immunol. 7 (6), 793–798.

Chen, K., Iribarren, P., Hu, J., Chen, J., Gong, W., Cho, E.H., Lockett, S., Dunlop, N.M., Wang, J.M., 2006. Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid beta peptide. J. Biol. Chem. 281 (6), 3651–3659.

Dahlberg, A., Auble, M.R., Petro, T.M., 2006. Reduced expression of IL-12 p35 by SJL/J macrophages responding to Theiler's virus infection is associated with constitutive activation of IRF-3. Virology 353 (2), 422–432.

Devergne, O., Hummel, M., Koeppen, H., Le Beau, M.M., Nathanson, E.C., Kieff, E., Birkenbach, M., 1996. A novel interleukin-12 p40-related protein induced by latent Epstein-Barr virus infection in B lymphocytes. J. Virol. 70 (2), 1143–1153.

Devergne, O., Birkenbach, M., Kieff, E., 1997. Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. Proc. Natl. Acad. Sci. USA 94 (22), 12041–12046.

Dong, C., Davis, R.J., Flavell, R.A., 2002. MAP kinases in the immune response. Annu. Rev. Immunol. 20, 55–72.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian. Cells 411 (6836), 494–498.

Goldberg, R., Zohar, Y., Wildbaum, G., Geron, Y., Maor, G., Karin, N., 2004. Suppression of ongoing experimental autoimmune encephalomyelitis by neutralizing the function of the p28 subunit of IL-27. J. Immunol. 173 (10), 6465–6471.

Gorden, K.K.B., Qiu, X.X., Binsfeld, C.C.A., Vasilakos, J.P., Alkan, S.S., 2006. Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and PolyT oligodeoxynucleotides. J. Immunol. 177 (10), 6584–6587.

Haahr, S., Plesner, A.M., Vestergaard, B.F., Hollsberg, P., 2004. A role of late Epstein-Barr virus infection in multiple sclerosis. Acta Neurol. Scand. 109 (4), 270–275.

Hafler, D.A., 2004. Multiple sclerosis. J. Clin. Invest. 113 (6), 788–794.

Heil, F., Ahmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., Dietrich, H., Lipford, G., Takeda, K., Akira, S., Wagner, H., Bauer, S., 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur. J. Immunol. 33 (11), 2987–2997.

Heil, F., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526–1529.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., Akira, S., 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat. Immunol. 3 (2), 196–200.

Holloway, G., Coulson, B.S., 2006. Rotavirus activates JNK and p38 signaling pathways in intestinal cells leading to AP-1-driven transcriptional responses and enhanced virus replication. J. Virol. 80 (21), 10624–10633.

Hsieh, C.S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A., Murphy, K.M., 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260 (5107), 547–549.

Jiang, Z., Zamanian-Daryoush, M., Nie, H., Silva, A.M., Williams, B.R., Li, X., 2003. Poly(I-C)-induced Toll-like receptor 3 (TLR3)-mediated activation of NFkappa B and MAP kinase is through an interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. J. Biol. Chem. 278 (19), 16713–16719.

Jones, B.W., Means, T.K., Heldwein, K.A., Keen, M.A., Hill, P.J., Belisle, J.T., Fenton, M.J., 2001. Different Toll-like receptor agonists induce distinct macrophage responses. J. Leukoc. Biol. 69 (6), 1036–1044.

Kwon, D., Fuller, A.C., Palma, J.P., Choi, I.H., Kim, B.S., 2004. Induction of chemokines in human astrocytes by picornavirus infection requires activation of both AP-1 and NF-kappa B. Glia 45 (3), 287–296.

Lang, H.L., Jacobsen, H., Ikemizu, S., Andersson, C., Harlos, K., Madsen, L., Hjorth, P., Sondergaard, L., Svejgaard, A., Wucherpfennig, K., Stuart, D.I., Bell, J.I., Jones, E.Y., Fugger, L., 2002. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. Nat. Immunol. 3 (10), 940–943.

Levin, L.I., Munger, K.L., Rubertone, M.V., Peck, C.A., Lennette, E.T., Spiegelman, D., Ascherio, A., 2005. Temporal relationship between elevation of Epstein-Barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA 293 (20), 2496–2500.

Lipton, H.L., Twaddle, G., Jelachich, M.L., 1995. The predominant virus antigen burden is present in macrophages in Theiler's murine encephalomyelitis virus-induced demyelinating disease. J. Virol. 69 (4), 2525–2533.

Lipton, H.L., Kumar, A.S., Trottier, M., 2005. Theiler's virus persistence in the central nervous system of mice is associated with continuous viral replication and a difference in outcome of infection of infiltrating macrophages versus oligodendrocytes. Virus Res. 111 (2), 214–223.

McLean, T.I., Bachenheimer, S.L., 1999. Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication. J. Virol. 73 (10), 8415–8426.

Monteyne, P., Bihl, F., Levillayer, F., Brahic, M., Bureau, J.F., 1999. The Th1/Th2 balance does not account for the difference of susceptibility of mouse strains to Theiler's virus persistent infection. J. Immunol. 162 (12), 7330–7334.

Nguyen, K.B., Salazar-Mather, T.P., Dalod, M.Y., Van Deusen, J.B., Wei, X.Q., Liew, F.Y., Caligiuri, M.A., Durbin, J.E., Biron, C.A., 2002. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. J. Immunol. 169 (8), 4279–4287.

Olson, J.K., Girvin, A.M., Miller, S.D., 2001. Direct activation of innate and antigen-presenting functions of microglia following infection with Theiler's virus. J. Virol. 75 (20), 9780–9789.

Olson, J.K., Miller, S.D., 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J. Immunol. 173 (6), 3916–3924.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J.S., Moore, K.W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J.F., Kastelein, R.A., 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13 (5), 715–725.

Pahan, K., Sheikh, F.G., Liu, X., Hilger, S., McKinney, M., Petro, T.M., 2001. Induction of nitric-oxide synthase and activation of NF-kappaB by interleukin-12 p40 in microglial cells. J. Biol. Chem. 276 (11), 7899–7905.

Petro, T.M., 2005a. Disparate expression of IL-12 by SJL/J and B10.S macrophages during Theiler's virus infection is associated with activity of TLR7 and mitogen-activated protein kinases. Microbes Infect. 7 (2), 224–232.

Petro, T.M., 2005b. ERK-MAP-kinases differentially regulate expression of IL-23 p19 compared with p40 and IFN-beta in Theiler's virus-infected RAW264.7 cells. Immunol. Lett. 97 (1), 47–53.

Pflanz, S., Timans, J.C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W.M., Mattson, J.D., Wagner, J.L., To, W., Zurawski, S., McClanahan, T.K., Gorman, D.M., Bazan, J.F., de Waal Malefyt, R., Rennick, D., Kastelein, R.A., 2002. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity 16 (6), 779–790.

Rahaus, M., Desloges, N., Wolff, M.H., 2004. Replication of varicella-zoster virus is influenced by the levels of JNK/SAPK and p38/MAPK activation. J. Gen. Virol. 85 (Pt 12), 3529–3540.

Schijns, V.E., Haagmans, B.L., Horzinek, M.C., 1995. IL-12 stimulates an antiviral type 1 cytokine response but lacks adjuvant activity in IFN-gamma-receptor-deficient mice. J. Immunol. 155 (5), 2525–2532.

Shim, J.H., Xiao, C., Paschal, A.E., Bailey, S.T., Rao, P., Hayden, M.S., Lee, K.Y., Bussey, C., Steckel, M., Tanaka, N., Yamada, G., Akira, S., Matsumoto, K., Ghosh, S., 2005. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev. 19 (22), 2668–2681.

So, E.Y., Kang, M.H., Kim, B.S., 2006. Induction of chemokine and cytokine genes in astrocytes following infection with Theiler's murine encephalomyelitis virus is mediated by the Toll-like receptor 3. Glia 53 (8), 858–867.

Steer, S.A., Moran, J.M., Christmann, B.S., Maggi Jr., L.B., Corbett, J.A., 2006. Role of MAPK in the regulation of double-stranded RNA- and encephalomyocarditis virus-induced cyclooxygenase-2 expression by macrophages. J. Immunol. 177 (5), 3413–3420.

Stewart, M.J., Kulkarni, S.B., Meusel, T.R., Imani, F., 2006. c-Jun N-terminal kinase negatively regulates dsRNA and RSV induction of tumor necrosis factor-alpha transcription in human epithelial cells. J. Interferon Cytokine Res. 26 (8), 521–533.

Sundstrom, P., Juto, P., Wadell, G., Hallmans, G., Svenningsson, A., Nystrom, L., Dillner, J., Forsgren, L., 2004. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology 62 (12), 2277–2282.

Takeuchi, O., Hemmi, H., Akira, S., 2004. Interferon response induced by Toll-like receptor signalling. J. Endotoxin Res. 10 (4), 252–256.

Zhao, J., Kong, H.J., Li, H., Huang, B., Yang, M., Zhu, C., Bogunovic, M., Zheng, F., Mayer, L., Ozato, K., Unkeless, J., Xiong, H., 2006. IRF-8/interferon (IFN) consensus sequence-binding protein is involved in Toll-like receptor (TLR) signaling and contributes to the cross-talk between TLR and IFN-{gamma} signaling pathways. J. Biol. Chem. 281 (15), 10073–10080.