

Src family kinases participate in the regulation of encephalomyocarditis virus-induced cyclooxygenase-2 expression by macrophages

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Src family kinases (SFKs) are non-receptor tyrosine kinases that have been implicated as regulators of the inflammatory response. In this study, the role of SFK activation in the inflammatory response of macrophages to encephalomyocarditis virus (EMCV) infection was examined. Virus infection of macrophages stimulates the expression of cyclooxygenase-2 (COX-2), interleukin (IL)-1 β and inducible nitric oxide synthase (iNOS). Inhibition of SFK attenuates EMCV-induced COX-2 expression and prostaglandin E₂ production, iNOS expression and subsequent nitric oxide production, and IL-1 β expression. EMCV-induced COX-2 expression requires the activation of nuclear factor- κ B and the mitogen-activated protein kinase p38. Consistent with these previous findings, inhibition of SFKs attenuated the phosphorylation of p38 in response to EMCV infection, suggesting that SFKs may act upstream of p38. These findings provide evidence that SFK activation plays an active role in the regulation of inflammatory gene expression by virus-infected macrophages.

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INTRODUCTION

Macrophages play a crucial role in the inflammatory response to pathogens by producing inflammatory mediators, which recruit and activate a variety of cell types. The immune response of macrophages to pathogens is initiated by the recognition of pathogen-associated molecular patterns by pattern-recognition receptors (PRRs) (Medzhitov, 2007). Activation of these immune receptors initiates signalling pathways, resulting in the expression of inflammatory genes.

Src family kinases (SFKs) have been implicated in the regulation of the macrophage inflammatory response to various stimuli recognized by PRRs (Boulet *et al.*, 1992; English *et al.*, 1997; Leu *et al.*, 2006; Orlicek *et al.*, 1999; Stovall *et al.*, 2004; Ziegler *et al.*, 1988). SFKs are non-receptor tyrosine kinases important in the regulation of a variety of cellular processes including cell proliferation, survival and the inflammatory response (Lowell, 2004; Okutani *et al.*, 2006; Parsons & Parsons, 2004). Pharmacological inhibition of SFKs attenuates cyclooxygenase-2 (COX-2) expression and nitrite production by rat peritoneal macrophages in response to lipopolysaccharide, a ligand for the PRR Toll-like receptor

4 (TLR4) (Leu *et al.*, 2006). Furthermore, SFK inhibition attenuates tumour necrosis factor (TNF) production and inducible nitric oxide synthase (iNOS) expression by RAW264.7 murine macrophages in response to treatment with bacterial DNA mimetic CpG, a ligand for TLR9 (Stovall *et al.*, 2004). Whilst SFK activation appears to participate in the inflammatory gene response to a number of TLRs, little is known concerning the role of SFKs in regulating virus-induced inflammatory responses of infected macrophages.

Virus infection of macrophages stimulates an inflammatory response that is characterized by the expression of COX-2, iNOS and interleukin (IL)-1 β . COX-2 catalyses the oxidation of arachidonic acid to prostaglandin H₂, which is subsequently isomerized to various prostanoids, including prostaglandin E₂ (PGE₂) (Smith *et al.*, 2000). PGE₂ participates in the inflammatory response by enhancing the replication of many viruses, whilst inhibiting replication of other viruses (Steer & Corbett, 2003). The expression of iNOS and subsequent production of nitric oxide attenuates virus replication, in part, by nitrosylation and inactivation of viral proteins required for replication (Karupiah *et al.*, 1993; Saura *et al.*, 1999). IL-1 β participates in the inflammatory response through recruitment of immune cells and has been implicated in the pathophysiology of many inflammatory diseases (Arend *et al.*, 2008).

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In support of a regulatory role of SFKs in the inflammatory macrophage response to virus infection, recent studies have shown that SFK inhibition in dendritic cells results in attenuation of antiviral signalling in response to dsRNA, a viral replication intermediate (Johnsen *et al.*, 2006). Additionally, SFKs have been implicated in the regulation of TNF- α production in response to human immunodeficiency virus type 1 envelope protein in human macrophages (Tomkowicz *et al.*, 2006). In response to encephalomyocarditis virus (EMCV), Yoon and co-workers showed that intraperitoneal administration of the SFK inhibitor PP2 attenuated the accumulation of inflammatory cytokine mRNA in peritoneal macrophages (Choi *et al.*, 2001). These studies suggest a potential role for SFKs in antiviral signalling in macrophages.

In this study, we examined the role of SFKs in the regulation of COX-2 production by macrophages in response to EMCV, a picornavirus that has been used as a prototype virus to study antiviral responses (Iordanov *et al.*, 2000). Inhibition of SFKs attenuates EMCV-induced COX-2 expression and PGE₂ production, iNOS expression and subsequent nitric oxide production, and IL-1 β expression. We have shown previously that p38 activation is required for poly(I:C)- and EMCV-stimulated COX-2 expression, and we now provide evidence that SFKs may function upstream of mitogen-activated protein kinase (MAPK) p38 in the regulation of COX-2 expression. Our findings suggest a novel role of SFKs in the regulation of the inflammatory response of macrophages to virus infection.

RESULTS

SFK inhibition attenuates EMCV-induced COX-2 expression and PGE₂ production by macrophages

To examine whether SFKs participate in EMCV-induced COX-2 protein expression (Steer *et al.*, 2003), RAW264.7 cells were treated with EMCV for 24 h in the absence or presence of the SFK inhibitor PP2. As shown in Fig. 1(a), pre-treatment with 10 μ M PP2 resulted in ~90% inhibition of EMCV-induced COX-2 protein expression (determined by densitometry). PP3, an inactive analogue of PP2, did not modify EMCV-stimulated COX-2 expression by RAW 264.7 cells (Fig. 1a). SU6656 is a structurally distinct SFK inhibitor that also attenuated EMCV-induced COX-2 expression (Fig. 1b), providing evidence that two structurally different SFK inhibitors attenuate the stimulatory actions of EMCV on COX-2 expression. PP2 treatment also attenuated EMCV-induced COX-2 protein expression in primary mouse peritoneal macrophages (~80% inhibition; Fig. 1c). The inhibitory actions of PP2 on COX-2 expression appeared to be mediated by attenuation of mRNA accumulation. Similar to previous studies, EMCV stimulated the accumulation of COX-2 mRNA expression following a 6 h treatment (Steer *et al.*, 2006). Both PP2 and SU6656 reduced EMCV-stimulated COX-2

mRNA accumulation in RAW264.7 cells in a concentration-dependent manner (Fig. 1d). COX-2 catalyses the oxidation of arachidonic acid to prostaglandin H₂, which is subsequently isomerized to various prostanoids, including PGE₂ (Smith *et al.*, 2000). Consistent with the inhibition of COX-2 mRNA and protein expression, PP2 also attenuated EMCV-stimulated PGE₂ production by RAW264.7 cells. Following a 30 min pre-treatment, PP2 reduced EMCV-induced PGE₂ production by ~70% (Fig. 1e). These findings support a role for SFKs in regulating the expression of COX-2 in virus-infected macrophages.

To confirm the pharmacological approaches described in Fig. 1, molecular approaches were used to examine the role of SFKs in the regulation of COX-2 expression by macrophages. RAW264.7 cells were transiently transfected with a control vector or a vector encoding an inactive Src mutant that contains a K296R point mutation in the kinase domain and a Y528F mutation at the phosphorylation site that provides negative regulation of the kinase. There was attenuation in the stimulatory effects of EMCV on COX-2 expression in RAW 264.7 cells expressing this dominant-negative Src mutant compared with cells expressing the empty vector or control macrophages. EMCV-induced COX-2 protein accumulation was reduced by ~70% in RAW264.7 cells expressing dominant-negative Src plasmid (Fig. 2). These findings, which are consistent with the inhibitory actions of PP2 on EMCV-stimulated COX-2 expression, provide molecular evidence to support a role for SFK activation in the expression of COX-2 in EMCV-infected macrophages.

SFK inhibition attenuates EMCV-induced iNOS and IL-1 β expression by macrophages

In addition to COX-2 expression, the macrophage response to virus infection also includes the expression of IL-1 β and iNOS (Heitmeier *et al.*, 1998), and each of these inflammatory genes appears to be regulated by signalling pathways that are common to each gene product and also by pathways that are selective for the target gene of interest. Nuclear factor (NF)- κ B is the common signalling pathway that regulates each of these inflammatory genes, whilst the calcium-independent phospholipase A₂ and cAMP-response element-binding protein appear to be selective for iNOS (Maggi *et al.*, 2002), extracellular signal-regulated kinase (ERK) is selective for IL-1 β (Maggi *et al.*, 2003), and c-Jun N-terminal kinase (JNK) and p38 are selective for COX-2 expression (Steer *et al.*, 2006). To determine whether SFKs control the expression of inflammatory genes in addition to COX-2, the effects of EMCV infection on iNOS expression, nitric oxide production and IL-1 β expression were examined. EMCV-induced iNOS expression was attenuated by PP2 in a concentration-dependent manner with a reduction of ~90% at 10 μ M (Fig. 3a). Consistent with iNOS expression, PP2 inhibited EMCV-induced nitrite production in a concentration-dependent

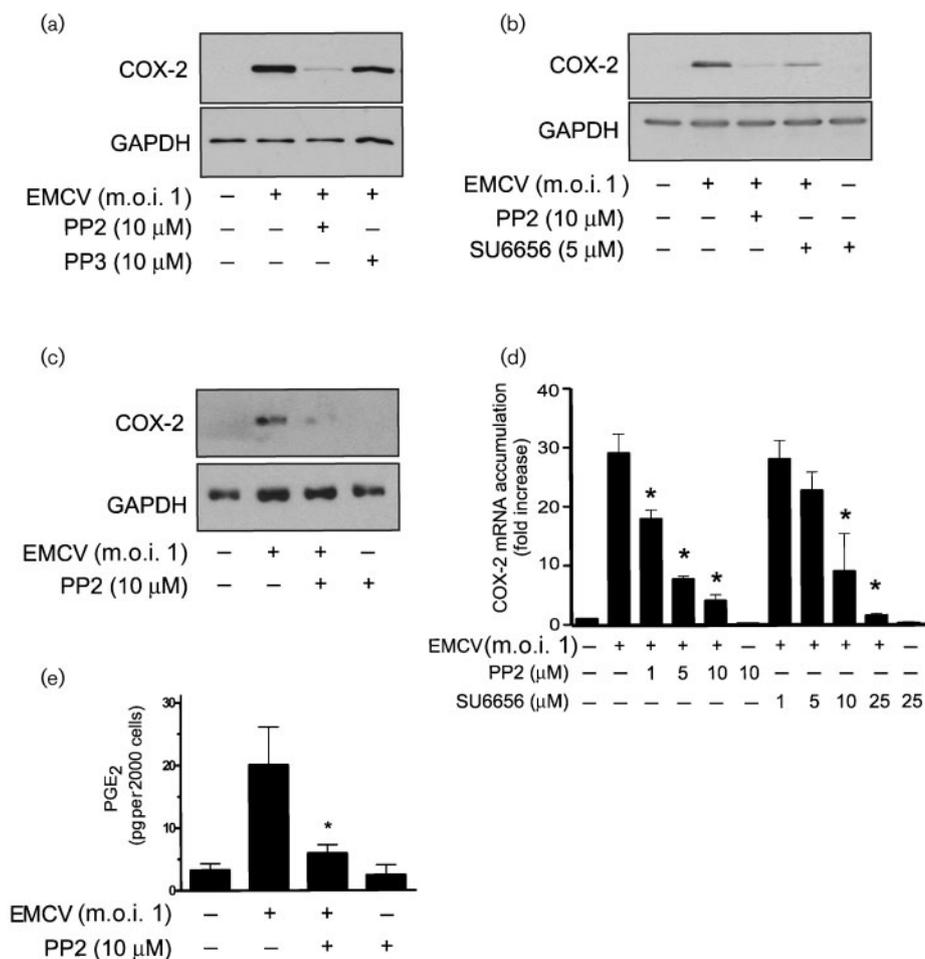


Fig. 1. SFK inhibition attenuates EMCV-induced COX-2 expression by macrophages. (a, b) RAW264.7 cells (1×10^5 in 400 μ l DMEM) were pre-treated with the indicated concentrations of PP2, PP3 or SU6656 for 30 min at 37 $^{\circ}$ C and cultured for an additional 24 h in the presence of EMCV (m.o.i. 1). COX-2 protein expression was determined by Western blot analysis. (c) Primary macrophages were isolated by peritoneal lavage, adjusted to a final concentration of 4×10^5 in 400 μ l complete CMRL-1066 and pre-treated for 30 min at 37 $^{\circ}$ C with 10 μ M PP2 as indicated. Macrophages were subsequently infected with EMCV (m.o.i. 1) for 24 h, and COX-2 protein expression was determined by Western blot analysis of whole-cell lysates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control in (a)–(c). (d) RAW264.7 cells (2×10^5 in 400 μ l DMEM) were pre-treated with PP2 or SU6656 for 30 min and infected with EMCV (m.o.i. 1) for 6 h, and total RNA was isolated. The accumulation of COX-2 mRNA was determined by real-time PCR and normalized to GAPDH mRNA levels. (e) RAW264.7 cells (2×10^5 in 400 μ l DMEM) were pre-treated with PP2 (10 μ M) for 30 min at 37 $^{\circ}$ C and then cultured for an additional 24 h in the presence of EMCV (m.o.i. 1). Cell culture supernatants were harvested and PGE₂ accumulation was determined by ELISA. The results are representative of two or three independent experiments (a–c) or the mean \pm SEM of three independent experiments (d, e). *, $P < 0.05$ compared with the EMCV-treated condition.

manner (Fig. 3a). Similar results for iNOS expression and nitrite production were observed using a second Src inhibitor, SU6656 (not shown). In addition to iNOS, PP2 also attenuated EMCV-induced pro-IL-1 β expression in peritoneal macrophages ($\sim 60\%$ reduction with 10 μ M PP2; Fig. 3b). Consistent with the inhibitor studies, transfection with the dominant-negative Src plasmid attenuated EMCV-induced pro-IL-1 β protein expression by RAW264.7 cells (not shown). These findings suggested that SFKs participate in the regulation of other EMCV-induced inflammatory genes, in addition to COX-2.

EMCV rapidly induces Src phosphorylation in macrophages

To confirm that EMCV activates SFKs in macrophages, EMCV-induced Src phosphorylation was examined at Y416, the autophosphorylation site that is indicative of activation (Lowell, 2004). As shown in Fig. 4, EMCV stimulated a rapid fourfold increase in Y416 phosphorylation of Src at 15 min post-infection, and the PP2 inhibitor prevented this EMCV-induced phosphorylation of Src. This rapid activation of Src by EMCV is consistent with the

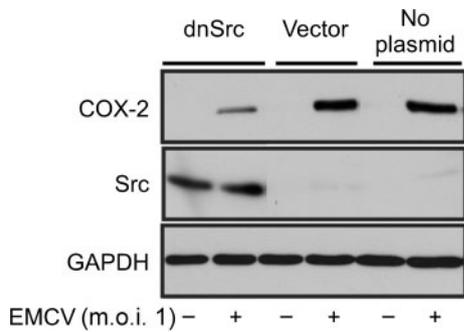


Fig. 2. Transient transfection with a dominant-negative Src attenuates EMCV-induced COX-2 expression by macrophages. RAW264.7 cells (2×10^6) were electroporated with 2 μ g dominant-negative Src plasmid (dnSrc) or a vector control, or were mock transfected. Transfected cells (4×10^5 in 400 μ l DMEM per condition) were treated with EMCV (m.o.i. 1) for 24 h. Total cell lysates were harvested and protein expression was determined by Western blot analysis. The results are representative of three independent experiments. GAPDH was used as a loading control.

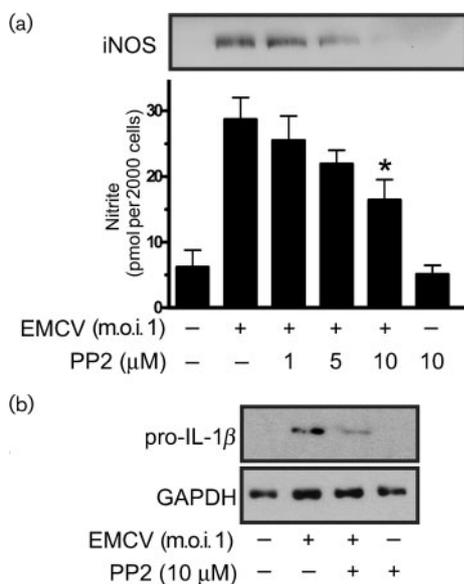


Fig. 3. SFK inhibition attenuates EMCV-induced iNOS and IL-1 β expression, and nitrite production by macrophages. (a) RAW264.7 cells (2×10^5 in 400 μ l DMEM) were pre-treated with the indicated concentrations of PP2 for 30 min at 37 $^{\circ}$ C and cultured for an additional 24 h in the presence of EMCV (m.o.i. 1). iNOS protein expression was determined by Western blot analysis, and nitrite production in the culture supernatant was quantified by a Greiss assay. (b) Peritoneal macrophages (4×10^5 in 400 μ l complete CMRL-1066) were pre-treated with 10 μ M PP2 for 30 min at 37 $^{\circ}$ C, cultured for 24 h in the presence of EMCV (m.o.i. 1), and pro-IL-1 β protein expression was determined by Western blot analysis. Results are representative of three independent experiments or represent the mean \pm SEM of four independent experiments. *, $P < 0.05$ compared with the EMCV-treated condition.

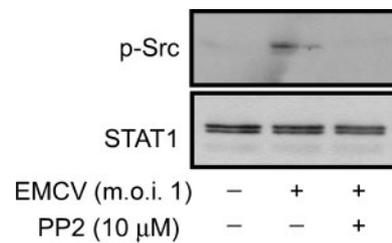


Fig. 4. EMCV infection of macrophages results in Src tyrosine kinase phosphorylation. RAW264.7 cells (2×10^5 in 400 μ l DMEM) were pre-treated with 10 μ M PP2 for 30 min at 37 $^{\circ}$ C and infected with EMCV (m.o.i. 1) for 15 min. Src phosphorylation was determined by Western blot analysis using a phospho-specific (Y416) antibody. Total signal transducer and activator of transcription 1 (STAT1) expression was determined as a loading control. The results are representative of three independent experiments.

rapid activation of NF- κ B and MAPK signalling pathways in macrophages in response to EMCV (Moran *et al.*, 2005). However, in contrast to the transient activation of MAPK by EMCV (Moran *et al.*, 2005), Src phosphorylation remained at an increased level 1 h after EMCV treatment (not shown). The prolonged increase in Src phosphorylation for greater than 1 h has been observed previously for other ligands, including dsRNA (Johnsen *et al.*, 2006) and granulocyte-macrophage colony-stimulating factor (Suh *et al.*, 2005).

SFKs are not required for EMCV-induced I κ B degradation

Activation of NF- κ B is required for expression of the inflammatory genes COX-2, IL-1 β and iNOS by macrophages in response to EMCV infection (Moran *et al.*, 2005; Steer *et al.*, 2003). As SFKs appear to participate in the regulation of these inflammatory genes, the potential role of SFKs in the activation of NF- κ B was examined. Using the degradation of I κ B α as an index for NF- κ B activation, we showed that inhibition of SFKs using PP2 did not attenuate EMCV-induced I κ B α degradation (Fig. 5a). We examined I κ B α expression at multiple time points after EMCV infection, and found no effect of PP2 on the kinetics of I κ B degradation (not shown). These findings suggest that SFKs do not regulate the inflammatory response by acting upstream of NF- κ B activation.

SFK inhibition attenuates EMCV-induced p38 phosphorylation

In addition to NF- κ B, EMCV rapidly activates the MAPK p38, JNK and ERK. Because p38 and JNK regulate EMCV-induced COX-2 expression by macrophages (Steer *et al.*, 2006), we hypothesized that SFKs may regulate COX-2 expression upstream of p38 and JNK. A 15 or 30 min infection with EMCV induced the phosphorylation of p38,

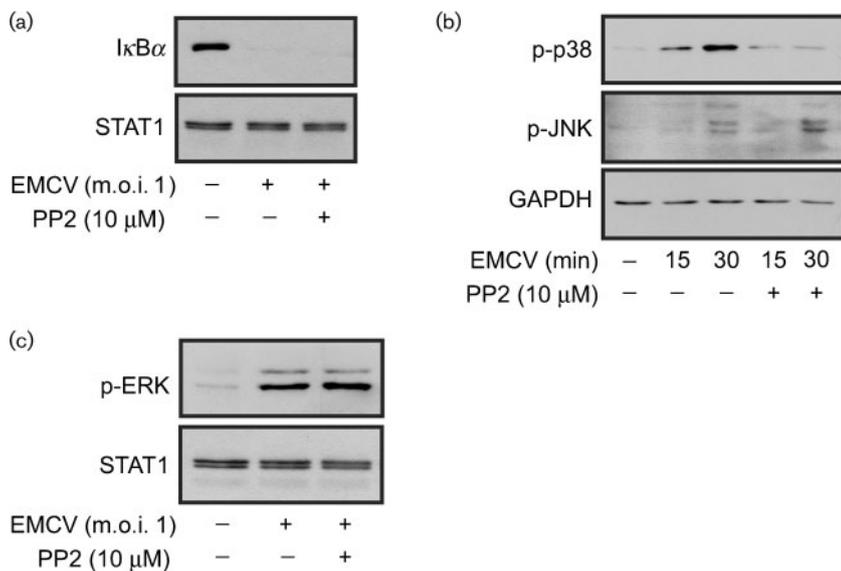


Fig. 5. IκBα degradation and MAPK phosphorylation. RAW264.7 cells (2×10^5 in 400 μl DMEM) were pre-treated with 10 μM PP2 and then infected with EMCV (m.o.i. 1) for 15 min (a, c) or for the indicated times (b). IκBα degradation (a) and MAPK phosphorylation (b, c) were examined by Western blot analysis. GAPDH and total STAT1 protein expression were determined as loading controls. The results are representative of three independent experiments.

JNK and ERK. Whilst PP2 attenuated EMCV stimulated p38 phosphorylation (~70 and ~80% inhibition at 15 and 30 min, respectively), it failed to attenuate ERK or JNK phosphorylation (Fig. 5b, c). These findings suggest that SFKs may regulate COX-2 expression by acting upstream of p38.

DISCUSSION

In this study, the role of SFKs in the inflammatory response of macrophages to EMCV was examined. We showed that chemical and molecular inhibition of SFKs attenuated EMCV-induced inflammatory gene expression by macrophages. Inhibition of SFKs attenuated EMCV-induced COX-2, IL-1β and iNOS expression, as well as PGE₂ and nitrite accumulation by macrophages. SFKs have been implicated in the regulation of the macrophage inflammatory response to various PRR ligands. Our current study extends the role for SFKs in the macrophage inflammatory response to include a role in the response to virus infection.

Recent studies have identified a number of pathways involved in the regulation of inflammatory gene expression in virus-treated macrophages. NF-κB plays a primary role in regulating macrophage expression of iNOS, COX-2 and IL-1β in response to EMCV (Heitmeier *et al.*, 1998; Maggi *et al.*, 2003; Steer *et al.*, 2003). SFKs have been shown to participate in cytokine-induced COX-2 expression in human epithelial cells by activating IκB kinase, resulting in IκBα degradation and NF-κB activation (Huang *et al.*, 2003). Although NF-κB signalling is required for EMCV-induced COX-2, IL-1β and iNOS expression by macrophages (Heitmeier *et al.*, 1998; Steer *et al.*, 2003), we showed that SFK inhibition does not appear to prevent EMCV-stimulated IκBα degradation. These findings suggest that SFKs do not act upstream of NF-κB. In addition to NF-κB, the activation of a secondary signalling pathway that is selective for the target gene of interest is also

required for EMCV-induced inflammatory gene expression by macrophages. These secondary signalling pathways include JNK and p38 activation for COX-2 (Steer *et al.*, 2006), ERK activation for IL-1β (Maggi *et al.*, 2003) and calcium-independent phospholipase A₂ for iNOS (Maggi *et al.*, 2002). The ability of PP2 to attenuate EMCV-stimulated p38 activation suggests that SFKs participate in EMCV-induced COX-2 expression through the activation of p38. SFK inhibition did not modify EMCV-stimulated JNK and ERK phosphorylation, suggesting that activation of these pathways by virus infection occurs by SFK-independent processes. In accordance with these findings, SFKs have been shown to act upstream of p38 in the activation of neutrophils (Mocsai *et al.*, 2000). At present, it is unclear how SFKs participate in the regulation of macrophage expression of iNOS and IL-1β in response to EMCV. Whilst we have previously identified a primary role for ERK in the regulation of IL-1β expression, and for NF-κB in the regulation of IL-1β and iNOS expression in response to EMCV, inhibition of SFKs does not modify activation of these pathways in response to EMCV infection, suggesting the participation of pathways in addition to NF-κB, ERK and SFKs. The proposed mechanism by which SFKs regulate EMCV-induced COX-2 expression is depicted in Fig. 6.

Infection of macrophages with EMCV results in the rapid phosphorylation of Src; however, the mechanisms by which SFKs are activated have yet to be determined. Whilst the dsRNA-dependent protein kinase R (PKR) initiates various antiviral responses in infected cells, a number of studies have shown that EMCV-stimulated MAPK activation and inflammatory gene expression do not require the presence of PKR (Moran *et al.*, 2005; Steer *et al.*, 2003). Recently, SFKs were shown to participate in antiviral signalling (interferon regulatory factor-3 activation and STAT1 phosphorylation) in dendritic cells by associating with TLR3 (Johnsen *et al.*, 2006). However, the inflammatory

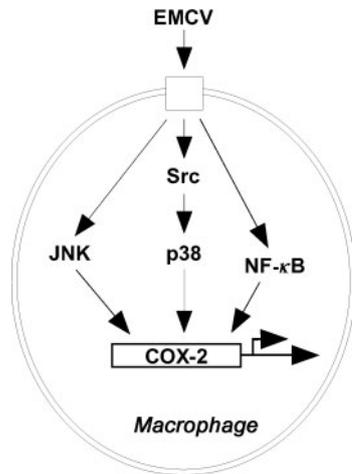


Fig. 6. Proposed mechanism by which SFKs regulate EMCV-induced COX-2 expression in macrophages.

response of macrophages to EMCV, including MAPK activation and COX-2 expression, occurs in the absence of the dsRNA receptor TLR3 (Steer *et al.*, 2006). Furthermore, the intracellular dsRNA receptor melanoma differentiation-associated gene 5 (mda5) (Gitlin *et al.*, 2006) does not appear to mediate the inflammatory response to EMCV (J. Corbett & B. Christmann, unpublished data). Thus, EMCV activates inflammatory pathways independent of PKR, TLR3 and mda5. The signalling receptor activated by EMCV is currently unknown. The intracellular dsRNA receptor retinoic acid inducible gene I (RIG-I) may mediate the EMCV-induced inflammatory response of macrophages (Yoneyama *et al.*, 2004). However, we believe that RIG-I is not a likely candidate, because EMCV capsid void of virus RNA is capable of activating MAPK and NF- κ B pathways and iNOS expression, suggesting that the signalling pathways regulating inflammatory gene expression do not require viral RNA accumulation or viral protein expression (Moran *et al.*, 2005). These findings suggest that EMCV capsid protein may activate signalling pathways by interacting with a cell-surface receptor. Consistent with this possibility, MAPK and NF- κ B signalling pathways, as well as SFKs, are rapidly activated after a 15 min EMCV infection. SFKs are known to be activated in response to stimulation of various cell-surface receptors, including G-protein-coupled receptors (GPCRs) (Thomas & Brugge, 1997). SFKs can be activated through the G-protein subunits $G\alpha_s$ and $G\alpha_i$ (Ma *et al.*, 2000), as well as independently of G proteins (McGarrigle & Huang, 2007; Sun *et al.*, 2007). We are currently examining the potential role of GPCRs as regulators of inflammatory gene expression by virus-infected macrophages.

METHODS

Materials and animals. RAW264.7 cells and L929 cells were obtained from the Washington University Tissue Culture Support

Center (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM; containing 10% heat-inactivated fetal calf serum and 1×10^{-6} M L-glutamine), CMRL-1066 tissue culture medium, L-glutamine, penicillin and streptomycin were from Gibco-BRL. C57BL/6J mice were purchased from Harlan. PP2, PP3 and SU6656 were purchased from Calbiochem. Rabbit anti-COX-2 and anti-iNOS antiserum were obtained from Cayman Chemicals. 3ZD monoclonal anti-pro-IL-1 β was obtained from the Biological Resources Branch at the NCI (National Institutes of Health, Bethesda, MD, USA), rabbit anti-phospho-ERK, anti-phospho-p38 and anti-phospho-JNK from Promega, rabbit anti-I κ B α and rabbit anti-STAT1 antiserum from Santa Cruz Biotechnology, mouse monoclonal anti-Src (clone GD11) from Upstate, rabbit anti-phospho-Src (Y416) from Cell Signaling Technology and mouse anti-GAPDH antiserum from Ambion. Horseradish peroxidase-conjugated donkey anti-rabbit and donkey anti-mouse antibodies were obtained from Jackson ImmunoResearch. The PCR primers for COX-2 and GAPDH were purchased from Integrated DNA Technologies. The dominant-negative Src in the pUSEamp expression vector was from Upstate.

Peritoneal macrophage isolation and cell culture. Primary macrophages were obtained from wild-type C57BL/6J mice by peritoneal lavage as described previously (Beckerman *et al.*, 1993). Briefly, the peritoneal cavity was injected with 10 ml ice-cold Hanks' balanced salt solution, the lavage was extracted and peritoneal exudate cells were collected by centrifugation, washed and plated at a density of 4×10^5 cells in 400 μ l complete CMRL-1066. Peritoneal cells were washed with CMLR-1066 to remove non-adherent cells.

RAW264.7 cells were removed from growth flasks by treatment with 0.05% trypsin/0.02% EDTA at 37 $^{\circ}$ C, washed with DMEM and plated at the indicated concentrations. Macrophages were cultured for a minimum of 2 h under an atmosphere of 95% air, 5% CO $_2$ prior to initiation of the experiments.

Virus propagation and infection. The B variant of EMCV was a generous gift from Dr Ji-Won Yoon (University of Calgary, Alberta, Canada) and has been described elsewhere (Bae *et al.*, 1989). EMCV was propagated in L929 cells, supernatants were clarified by centrifugation and titres were determined by plaque assay. Cell monolayers were infected at an m.o.i. of 1 by the addition of EMCV to the culture medium at 37 $^{\circ}$ C for the times indicated.

Western blot analysis. Protein samples were separated under denaturing conditions by SDS-PAGE and immobilized on nitrocellulose membranes (GE Healthcare) or PVDF membranes (Pall Life Sciences) under semi-dry transfer conditions. Antibody dilutions were as follows: rabbit anti-COX-2, 1:2000; mouse anti-pro-IL-1 β , 1:2000; mouse anti-GAPDH, 1:10 000; rabbit anti-phospho-p38, 1:4000; rabbit anti-phospho-ERK, 1:5000. All other primary antibodies were used at 1:1000. Horseradish peroxidase-conjugated donkey anti-mouse and donkey anti-rabbit secondary antibodies were used at 1:5000 and 1:7000 dilutions, respectively. Antigen was detected by chemiluminescence according to the manufacturer's specifications (GE Healthcare).

Real-time PCR. Total RNA was isolated from macrophages using an RNeasy kit according to the manufacturer's instructions (Qiagen). First-strand cDNA synthesis was performed using oligo(dT) and a reverse transcriptase Superscript pre-amplification system (Invitrogen) following the manufacturer's recommendations. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and a Research DNA Engine Opticon II thermocycler with continuous fluorescence detection (MJ Research). The mRNA levels of COX-2 were normalized to those of GAPDH. The PCR primer sequences for COX-2 were: 5'-TTTGTGAGTCATTCACCAGACAGAT-3' (forward) and 5'-CAGTATTGAGGAGAACAGATGGGATT-

3' (reverse). The PCR primers for GAPDH have been described elsewhere (Arnush *et al.*, 1998).

Transfection. RAW264.7 cells were transiently transfected using an Amaxa Nucleofector electroporator (Amaxa Biosystems). The cells were removed from growth flasks by treatment with 0.05 % trypsin/0.02 % EDTA at 37 °C, washed with DMEM and incubated for 2 h at 37 °C. The cells (2×10^6) were resuspended in electroporation buffer and electroporated with 2 µg plasmid using program D-032. A total of 4×10^5 cells in 400 µl DMEM were plated per condition. Six hours after transfection, the medium was replaced and the cells were cultured overnight at 37 °C. The cells were washed twice with PBS before initiation of experiments. We obtained a transfection efficiency of ~50 %, as determined by electroporation with a plasmid expressing enhanced green fluorescent protein.

Determination of nitrite and PGE₂. Nitrite production was determined by the addition of 50 µl Greiss reagent to 50 µl macrophage cell culture supernatant (Green *et al.*, 1982). Absorbance at 540 nm was measured and nitrite concentrations were quantified by comparison to a sodium nitrite standard curve. PGE₂ release in supernatants was determined using a PGE₂ enzyme immunoassay according to the manufacturer's specifications (Cayman Chemicals).

Statistics. Statistical comparisons between groups were made using one-way analysis of variance. Significant differences between groups ($P < 0.05$) were determined by Newman-Keuls post-hoc analysis. Western blots were quantified by densitometry using ImageQuant software (GE Healthcare) and values were normalized to loading controls.

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