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Activation of p38 MAPK by feline infectious peritonitis virus regulates pro-inflammatory cytokine production in primary blood-derived feline mononuclear cells

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ABSTRACT

Feline infectious peritonitis (FIP) is an invariably fatal disease of cats caused by systemic infection with a feline coronavirus (FCoV) termed feline infectious peritonitis virus (FIPV). The lethal pathology associated with FIP (granulomatous inflammation and T-cell lymphopenia) is thought to be mediated by aberrant modulation of the immune system due to infection of cells such as monocytes and macrophages. Overproduction of pro-inflammatory cytokines occurs in cats with FIP, and has been suggested to play a significant role in the disease process. However, the mechanism underlying this process remains unknown. Here we show that infection of primary blood-derived feline mononuclear cells by FIPV WSU 79-1146 and FIPV-DF2 leads to rapid activation of the p38 MAPK pathway and that this activation regulates production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-alpha) and interleukin-1 beta (IL-1 beta). FIPV-induced p38 MAPK activation and pro-inflammatory cytokine production was inhibited by the pyridinyl imidazole inhibitors SB 203580 and SC 409 in a dose-dependent manner. FIPV-induced p38 MAPK activation was observed in primary feline blood-derived mononuclear cells individually purified from multiple SPF cats, as was the inhibition of TNF-alpha production by pyridinyl imidazole inhibitors.

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Introduction

Coronaviruses are a diverse family of enveloped positive-stranded RNA viruses that infect a wide range of species including humans. Coronaviruses are divided into three groups in which group 1 and 2 infect mammals and group 3 infects birds (Perlman et al., 2008). Feline coronaviruses (FCoVs) belongs to group 1 and are classified as either serotype I or II depending on the sequence of their spike (S) protein (Rottier, 1999). In addition, each serotype is divided into two biotypes designated as either feline enteric coronavirus (FECV) or feline infectious peritonitis virus (FIPV) based on their pathological outcome in cats (Vennema et al., 1998). FECV is ubiquitous amongst felines and causes mild to often unapparent enteritis, while FIPV leads to a lethal systemic infection marked by severe granulomatous inflammation (Pedersen et al., 1984a, 1984b; Weiss and Scott, 1981). The mechanism underlying this drastic difference in disease between the two biotypes remains elusive, namely because FECV and FIPV isolates from the same serotype are virtually indistinguishable on the genetic and antigenic level. However it has been shown that the two biotypes possess markedly different abilities to infect cells of the immune

system, with FIPV isolates possessing an extended tropism that allows for the infection of macrophages and monocytes (Stoddart and Scott, 1989). Recent studies have suggested that this alteration in tropism may be due to mutations in the S protein that affect protein cleavage and fusion activation during entry (Regan et al., 2008; Rottier et al., 2005).

Viral pathogens that infect immune cells (e.g. human immunodeficiency virus (HIV) and Dengue Virus) are known to induce aberrant cytokine production, a process which is proposed to play a role in the pathological outcome of their respective diseases (Fantuzzi et al., 2003; Kedzierska and Crowe, 2002; Leong et al., 2007). Studies of cats with FIP have shown that cytokine expressions are altered as compared to healthy animals (Dean et al., 2003; Kiss et al., 2004). Specifically it has been noted that expression of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-alpha), interleukin-1 beta (IL-1 beta) and interleukin-6 (IL-6) are significantly increased in cats with FIP, and are likely produced by infected macrophages and monocytes (Kiss et al., 2004; Takano et al., 2007a, 2007b). It has been shown that TNF-alpha is able to induce feline T-cell apoptosis, making it the most likely causative agent of T-cell lymphopenia in FIPV-infected cats (Dean et al., 2003; Takano et al., 2007a). In addition TNF-alpha has been shown to increase expression of the FCoV receptor aminopeptidase N (APN) causing target cells to be more susceptible to viral infection and further exacerbate the disease (Takano et al.,

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2007b). However despite their critical role in the pathological outcome of FIP, the mechanism regulating FIPV-induced upregulation of pro-inflammatory cytokines remains undescribed.

Mitogen-activated protein kinases (MAPKs) are a family of proteins that serve as components of signaling pathways within cells in order to process and respond to extracellular stimuli (Raman et al., 2007). Typically, receptors on the cell surface initiate signaling cascades, which lead to phosphorylation and translocation of MAPKs to the nucleus where they regulate transcriptional activators (Whitmarsh, 2007). In recent years, it has become clear that MAPKs also regulate processes outside of the nucleus such as mRNA translation and cytoskeletal remodeling (Frevel et al., 2003; Huang et al., 2004). Three major MAPK pathways have been identified which are conserved in all eukaryotic cells ranging from yeast to mammals. These pathways are designated as Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2), c-Jun N-terminal Kinases (JNK1) and p38 MAPK (Pearson et al., 2001). In general the ERK pathway is activated by proliferative stimuli, while the JNK and p38 MAPK pathways are activated by extracellular stresses such as ultraviolet light, heat and osmotic shock (Pearson et al., 2001). p38 MAPK was originally identified as the target of pyridinyl imidazole compounds that were shown to inhibit the production of IL-1 and TNF- α in lipopolysaccharide (LPS)-stimulated human monocytes (Lee et al., 1994). Subsequent studies have shown that the p38 MAPK pathway is responsible for the phosphorylation of a large group of transcriptional and translational response elements which directly regulate the expression of a wide variety of pro-inflammatory cytokines (Kumar et al., 2003).

Due to its involvement in cytokine regulation, we reasoned that the p38 MAPK pathway might play a role in the increased production of pro-inflammatory cytokines observed in cats with FIP. In this study we examined the activation of the p38 MAPK pathway in response to infection by FIPV in primary feline blood-derived mononuclear cells. We also investigated the role of p38 MAPK in TNF- α , IL-1 beta and IL-6 production, and the effect of p38 MAPK inhibitors on these processes.

Results

Activation of the p38 MAPK pathway during FIPV infection

The p38 MAPK pathway has been shown to be activated by multiple viral pathogens during infection (Adamson et al., 2000; Banerjee et al., 2002; Dumitru et al., 2006; Erhardt et al., 2002; Holloway and Coulson, 2006; Zachos et al., 1999). To determine whether the p38 MAPK pathway is activated during the infectious life-cycle of FIPV, primary feline blood-derived mononuclear (PFBM) cells were inoculated with either FIPV-1146 or FIPV-DF2 at an MOI of 100. Untreated cells and infected cells ranging from 15 min to 12 h p.i. were lysed and analyzed by western blot with the anti-phospho-p38 MAPK mAb (3D7). Untreated cells showed a minimal level of p38 MAPK phosphorylation, however addition of either virus isolate caused rapid phosphorylation of p38 MAPK (>600% increase) within 15 min p.i. (Figs. 1A and C). p38 MAPK underwent dephosphorylation by 60 min and then showed a second phase of phosphorylation later in infection between 6 and 12 h p.i., which was less pronounced (Fig. 1A). To determine whether viral replication was required for FIPV-induced p38 MAPK activation, UV-inactivated virus was added to PFBM cells and analyzed by western blot as described above (Fig. 1B). UV-treated FIPV also induced p38 MAPK phosphorylation, however the activation was not biphasic, and instead remain sustained throughout the 12 h time-course (Fig. 1B). Membranes were re-probed with anti-p38 MAPK (N-20) pAb to show that an equal amount of p38 MAPK was present in each sample (Figs. 1A and B). To further confirm that the p38 MAPK pathway is activated by FIPV, PFBM cells were inoculated with FIPV-1146 at an MOI of 100 before fixing the cells for immunofluorescent microscopy. p38 MAPK again showed a rapid

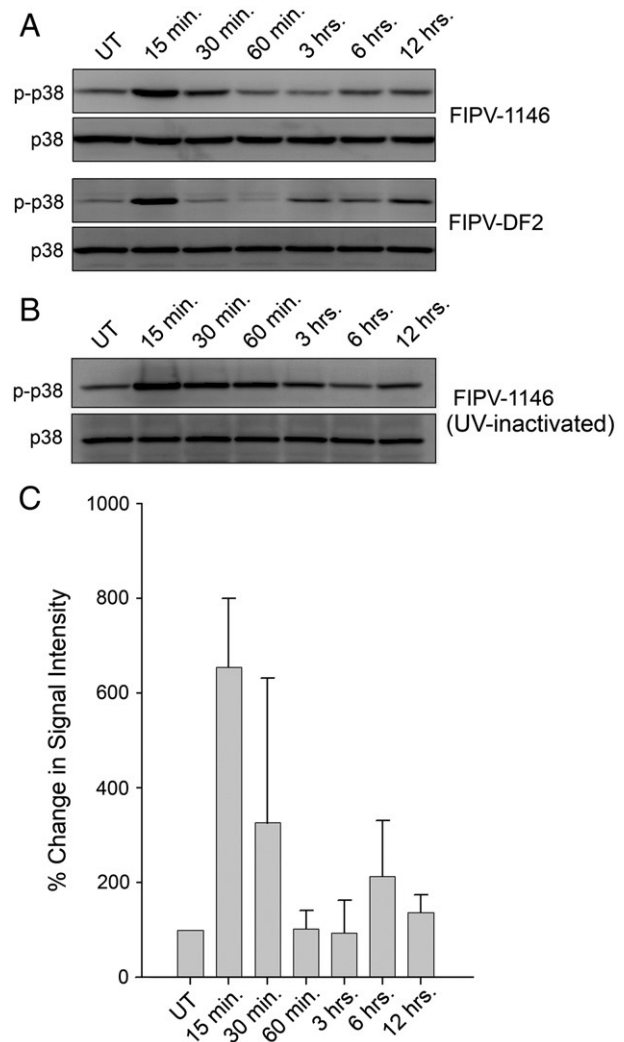


Fig. 1. FIPV induces phosphorylation of p38 MAPK in PFBM cells. PFBM cells were infected with either untreated FIPV-1146 or FIPV-DF2 viral particles (A), or UV-inactivated particles (B), at an MOI of 100. Cells were lysed at the specified times p.i. and analyzed by western blot with the anti-phospho-p38 MAPK mAb (3D7) and re-probed with anti-p38 MAPK (N-20) pAb. Signal intensity from two time-course infections of untreated FIPV-1146 and one time-course infection of untreated FIPV-DF2 were quantified by densitometry analysis with Image J software (C).

phosphorylation by 15 min p.i. while the total amount of p38 MAPK remained unchanged (Fig. 2). In addition the p38 MAPK in infected cells showed increased nuclear localization as compared to untreated cells, a phenomenon highly associated with the regulation of transcriptional activators (Fig. 2). These data indicate that the p38 MAPK pathway is activated during infection of PFBM cells by FIPV, and that viral replication is dispensable for this activation to occur.

Effect of pyridinyl imidazole inhibitors on FIPV-induced p38 MAPK activation

The p38 MAPK pathway was first discovered by investigating the target of pyridinyl imidazole compounds which blocked LPS-induced cytokine induction in human monocytes (Lee et al., 1994). To test the effect of pyridinyl imidazole inhibitors on FIPV-induced p38 MAPK phosphorylation, PFBM cells were treated with 10 μ M of either SB 203580 or SC 409 (or 0.1% DMSO as a control) for 2 h before inoculating with FIPV-1146 or FIPV-DF2 at an MOI of 100. 15 min p.i. cells were lysed and analyzed by western blot with the anti-phospho-p38 MAPK mAb (3D7). Cells which were pretreated with DMSO alone

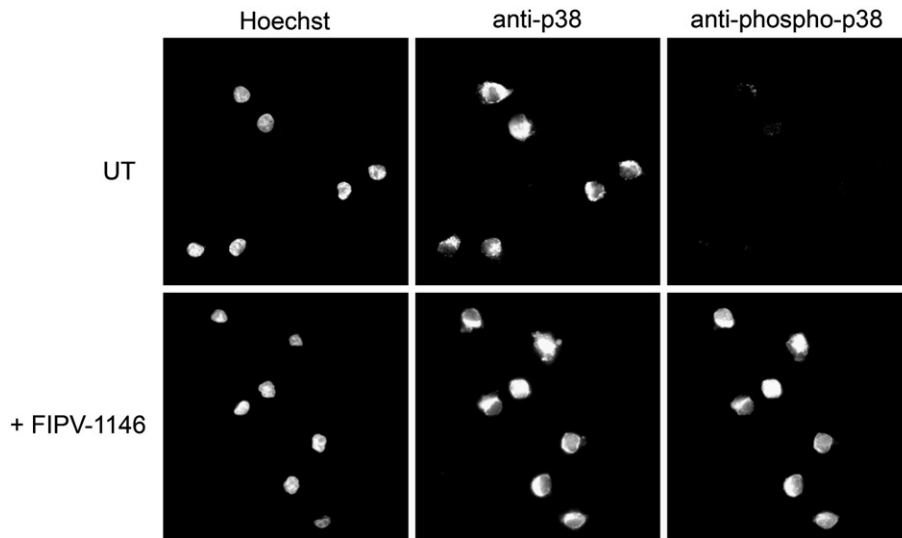


Fig. 2. Immunofluorescence of FIPV-induced p38 MAPK activation and nuclear re-localization. PFBM cells were infected with FIPV-1146 at an MOI of 100. Cells were fixed at 15 min p.i. and stained with the anti-phospho-p38 MAPK mAb (3D7), the anti-p38 MAPK (N-20) pAb and Hoechst.

showed rapid FIPV-induced phosphorylation of p38 MAPK, however those which were pretreated with either SB 203580 or SC 409 showed no activation as compared to uninfected cells (Fig. 3). These data demonstrate that FIPV-induced p38 MAPK activation is blocked by pyridinyl imidazole inhibitors.

FIPV replication is not affected by inhibition of the p38 MAPK pathway

Activation of the p38 MAPK pathway has been shown to be required for replication of some viruses including the murine coronavirus Mouse Hepatitis Virus (MHV) (Banerjee et al., 2002). To investigate whether activation of the p38 MAPK pathway is required for replication of FIPV, PFBM cells were treated with 10 μ M of either SB 203580 or SC 409 (or 0.1% DMSO as a control) for 2 h before inoculating with FIPV-1146 or FIPV-DF2. 12 h p.i. cells were fixed and stained for with the anti-FIPV N protein mAb (17B7.1). As shown in Fig. 4, pretreatment with p38 MAPK inhibitors has no significant effect on FIPV replication in PFBM cells.

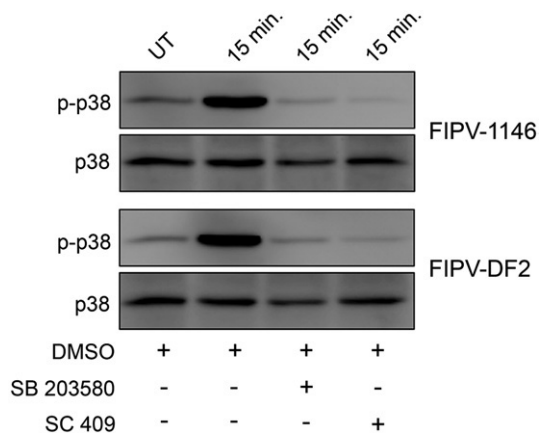


Fig. 3. Inhibition of FIPV-induced p38 MAPK activation in PFBM cells by the pyridinyl imidazole compounds SB 203580 and SC 409. PFBM cells were pretreated with 10 μ M of either SB 203580 or SC 409 (or 0.1% DMSO as a control) for 2 h before inoculating with FIPV-1146 or FIPV-DF2 at an MOI of 100. 15 min p.i. cells were lysed and analyzed by western blot with the anti-phospho-p38 MAPK mAb (3D7) and re-probed with anti-p38 MAPK (N-20) pAb.

TNF-alpha production is regulated by FIPV-induced p38 MAPK activation

Activation of p38 MAPK by viral pathogens has been shown to induce the production of pro-inflammatory cytokines such as TNF-alpha, IL-1 beta and IL-6 (Banerjee et al., 2002; Griego et al., 2000; Lee et al., 2005a, 2005b; Sloan and Jerome, 2007; Wang et al., 2004; Yurochko and Huang, 1999). To investigate whether TNF-alpha production in FIPV-infected PFBM cells is regulated by p38 MAPK activation, PFBM cells were treated with 10 μ M of either SB 203580, SC 409 or 0.1% DMSO for 2 h before inoculating with FIPV-1146 or FIPV-DF2 at an MOI of 100. 24 h p.i. supernatants were collected, concentrated and analyzed by western blot with the anti-TNF-alpha (N-19) pAb. Cells which were pretreated with DMSO alone showed significant production of TNF-alpha, however those which were pretreated with either SB 203580 or SC 409 showed no detectable production of TNF-alpha as compared to uninfected cells (Fig. 5A). To quantify the production of TNF-alpha, infections were performed as described above, except at 24 h p.i. supernatants were collected and analyzed by anti-TNF-alpha capture ELISA. Infected cells which were pretreated with DMSO alone showed significant production of TNF-alpha (>750 pg/ml) however pretreatment with 10 μ M SB 203580 and 10 μ M SC 409 resulted in a 8-fold and 4-fold reduction in TNF-alpha production respectively (Fig. 5B). Uninfected cells produced no TNF-alpha, or were below the detection level of the assay (data not shown). Overall these data indicate that production of the pro-inflammatory cytokine TNF-alpha in FIPV-infected PFBM cells is regulated by activation of the p38 MAPK pathway.

p38 MAPK inhibitors reduce TNF-alpha production in a dose-dependent manner

To show that the reduction of FIPV-induced TNF-alpha production by SB 203580 and SC 409 was specific to the pyridinyl imidazole inhibitors, PFBM cells were treated with either SB 203580 or SC 409 at a range of concentrations (10 μ M, 1 μ M or 0.1 μ M) or 0.1% DMSO for 2 h before inoculating with FIPV-1146 or FIPV-DF2 at an MOI of 100. As seen in our previous data, PFBM cells which were pretreated with DMSO alone produced significant amounts of TNF-alpha however pretreatment with SB 203580 and SC 409 resulted in a significant reduction in TNF-alpha production in a dose-dependent manner (Fig. 6).

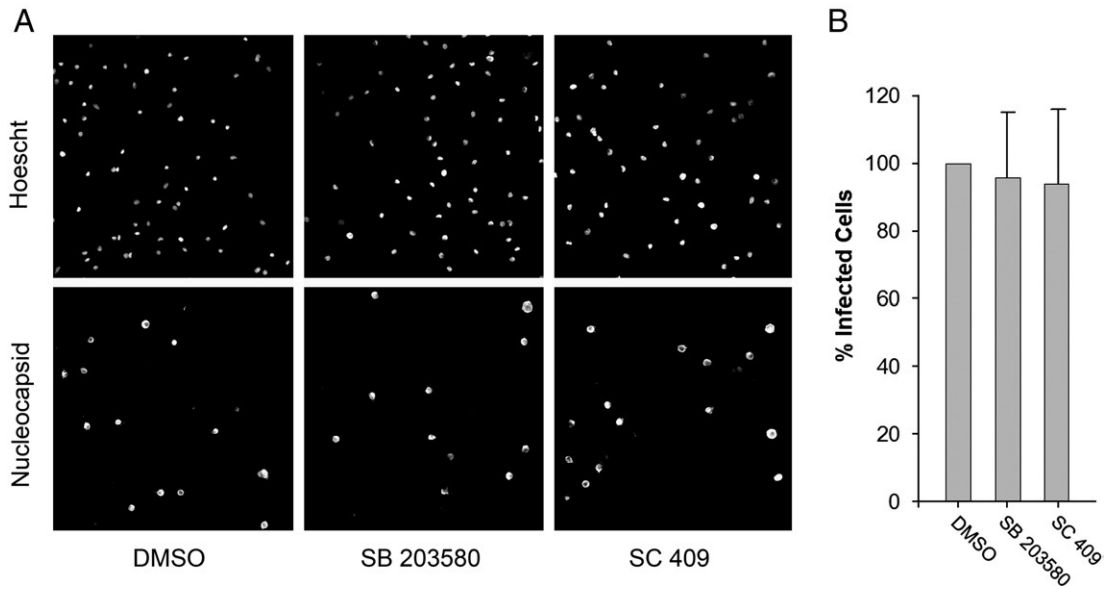


Fig. 4. Inhibition of the p38 MAPK pathway does not significantly affect FIPV infection of PFBM cells. PFBM cells were pretreated with 10 μ M of either SB 203580 or SC 409 (or 0.1% DMSO as a control) for 2 h before inoculating with FIPV-1146 or FIPV-DF2. Cells were fixed 12 h p.i. and stained with the anti-FIPV-N protein mAb (17B7.1) (A). For quantification, >500 cells were scored from three independent replicates of each experimental condition (B). Error bars represent the standard deviation of the mean.

FIPV-induced p38 MAPK activation regulates TNF-alpha production in multiple cats

It is known that individual animals can vary in their reaction to infection by FIPV (Kiss et al., 2004). To determine whether or not FIPV-induced p38 MAPK activation was specific to a single animal, PFBM cells were individually prepared from six additional SPF cats (07PGP2, 07PGV4, 07PGV5, 07FGR2, 07FGV6, 07FJM5). PFBM cells individually purified from each animal were inoculated with FIPV-1146 at an MOI of 100. Untreated cells and infected cells (15 min p.i.) were lysed and

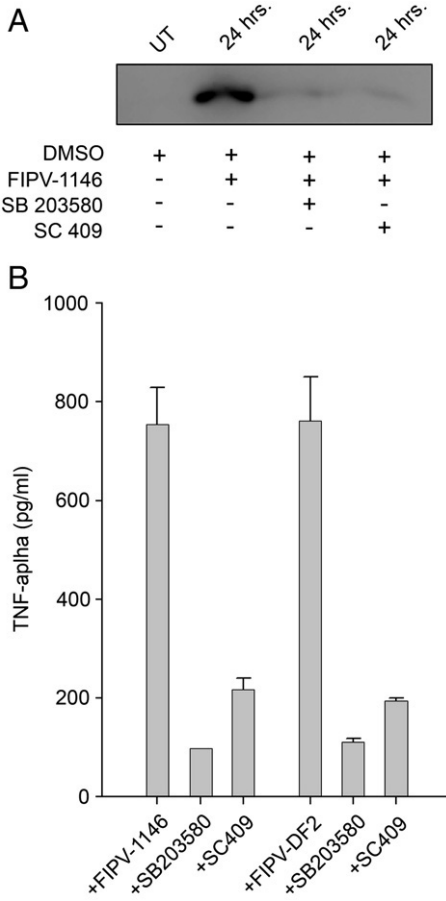


Fig. 5. FIPV-induced TNF-alpha production by PFBM cells is regulated by p38 MAPK activation. PFBM cells were pretreated with 10 μ M of either SB 203580 or SC 409 (or 0.1% DMSO as a control) for 2 h before inoculating with FIPV-1146 or FIPV-DF2 at an MOI of 100. 24 h p.i. supernatants were collected, concentrated and analyzed by western blot with the anti-TNF-alpha (N-19) pAb (A). 24 h p.i. supernatants were collected and TNF-alpha production was quantified by anti-TNF-alpha capture ELISA (B). TNF-alpha produced from untreated cells was below the detection limit of the assay (<10 pg/ml).

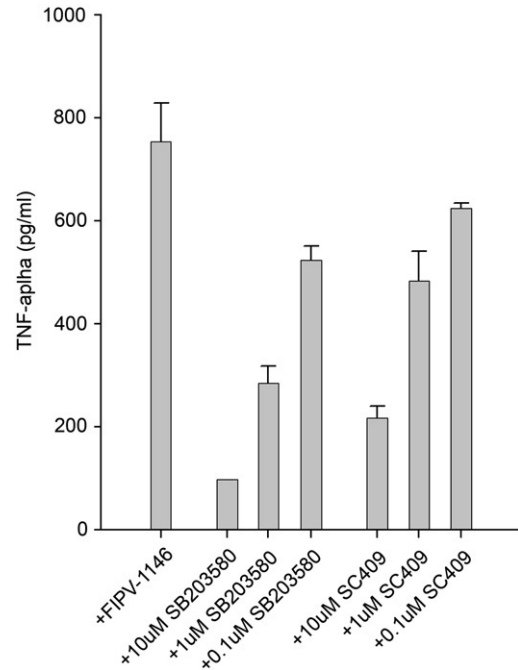


Fig. 6. FIPV-induced TNF-alpha production is inhibited in a dose-dependent manner by SB 203580 and SC 409. PFBM cells were pretreated with either SB 203580 or SC 409 at a range of concentrations (10 μ M, 1 μ M or 0.1 μ M) or 0.1% DMSO for 2 h before inoculating with FIPV-1146 at an MOI of 100. 24 h p.i. supernatants were collected and TNF-alpha production was quantified by anti-TNF-alpha capture ELISA. TNF-alpha from untreated cells was below the detection limit of the assay (<10 pg/ml).

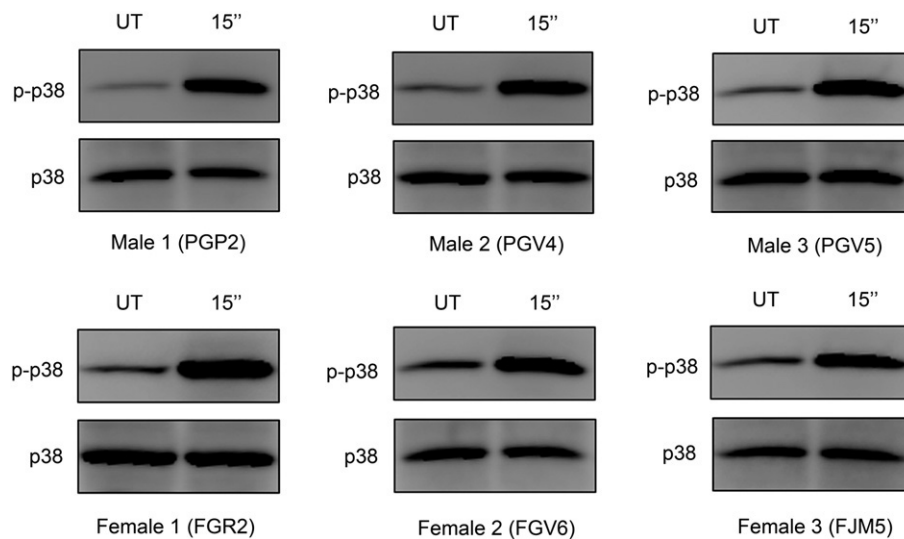


Fig. 7. FIPV-induced p38 MAPK activation in PFBM cells from six individual SPF cats. PFBM cells were individually prepared from three male (07PGP2, 07PGV4, 07PGV5) and three female (07FGR2, 07FGV6, 07FJM5) SPF cats. Cells from each animal were inoculated with FIPV-1146 at an MOI of 100. 15 min p.i. cells were lysed and analyzed by western blot with the anti-phospho-p38 MAPK mAb (3D7) and re-probed with anti-p38 MAPK (N-20) pAb.

analyzed by western blot with the anti-phospho-p38 MAPK mAb (3D7). Consistent with our previous data, untreated cells showed a minimal level of p38 MAPK phosphorylation while addition of FIPV caused a rapid phosphorylation of p38 MAPK in PFBM cells from all six cats (Fig. 7). Membranes were re-probed with anti-p38 MAPK (N-20) pAb to show that an equal amount of p38 MAPK was present in each sample (Fig. 7). In addition, the regulation of TNF-alpha production by p38 MAPK was analyzed in PFBM cells from all six SPF cats. PFBM cells from each animal were treated with 10 μ M SC 409 or 0.1% DMSO for 2 h before inoculating with FIPV-1146 at an MOI of 100. 24 h p.i. supernatants were collected and analyzed by anti-TNF-alpha capture ELISA. While the baseline level of TNF-alpha production differed slightly amongst all of the cats tested, treatment with the p38 inhibitor SC 409 resulted in the same trend observed in our previous experiments: a significant reduction in TNF-alpha levels (between 3-fold to 8-fold) (Fig. 8). These data taken together suggest that FIPV-induced activation of the p38 MAPK pathway in PFBM cells represents a common mechanism by which this virus promotes TNF-alpha production in cats.

IL-1 beta production is regulated by FIPV-induced p38 MAPK activation

Cats with FIP have also been reported to show increased levels of the pro-inflammatory cytokines IL-1 beta and IL-6. To investigate whether IL-1 beta and IL-6 production in FIPV-infected PFBM cells is regulated by p38 MAPK activation, PFBM cells were treated with 10 μ M of either SB 203580, SC 409 or 0.1% DMSO for 2 h before inoculating with FIPV-1146 at an MOI of 100. 24 h p.i. supernatants were collected and analyzed by anti-IL-1 beta and anti-IL-6 capture ELISA. Infected cells which were pretreated with DMSO alone showed significant production of IL-1 beta (>200 pg/ml) however pretreatment with 10 μ M SB 203580 and 10 μ M SC 409 resulted in a 7-fold and 4-fold reduction in IL-1 beta production respectively (Fig. 9). Neither infected nor uninfected PFBM cells produced significant levels of IL-6 (Fig. 9). Overall, these data indicate that both TNF-alpha and IL-1 beta production in FIPV-infected PFBM cells is regulated by p38 MAPK activation, a situation that does not apply to IL-6.

Discussion

Modulation of signaling pathways by viruses is becoming recognized as a key pathogenic determinant in viral diseases mediated

by aberrant host immunological responses. In the case of FIP, cytokine production is markedly altered between animals with disease as compared to healthy animals, with overproduction of the pro-inflammatory cytokine TNF-alpha in particular being indicative of a poor outcome (Kiss et al., 2004). Feline TNF-alpha causes apoptosis in feline T-cells (implicating it as the causative agent of T-cell lymphopenia), and upregulates the FIPV receptor APN making target cells more susceptible to infection in vitro (Dean et al., 2003; Kiss et al., 2004; Takano et al., 2007a, 2007b). It has been shown previously that FIPV-infected monocytes upregulate the expression of TNF-alpha, however the mechanism regulating this process remains undescribed. In this study we show that infection by FIPV causes a rapid activation the p38 MAPK pathway in PFBM cells, and that this process directly regulates production of the pro-inflammatory cytokines TNF-alpha and IL-1 beta.

As shown in Fig. 1, FIPV-induced p38 MAPK activation in PFBM cells occurs in a biphasic temporal pattern which mimics that observed with other viral pathogens that activate MAPK pathways during infection such as influenza virus (Pleschka et al., 2001). At present we are unable to define the mechanism by which FIPV particles are able to activate the p38 MAPK pathway, however the rapid nature of the initial activation suggests that it occurs early during entry; likely due to interactions between the S protein and its receptor. This model is further supported by the observation that UV-inactivated virus also induce rapid activation of the p38 MAPK pathway. This activation is markedly different than that reported in MHV infected cells, where activation did not occur until 6–12 h p.i., and UV-treated viral particles did not induce phosphorylation of p38 MAPK (Banerjee et al., 2002). It notable that the FIPV receptor APN localizes to lipid rafts (Navarrete Santos et al., 2000; Nomura et al., 2004) which are known to be a signaling portal for the p38 MAPK pathway (Calzolari et al., 2006; Head et al., 2006; Olsson and Sundler, 2006; Sugawara et al., 2007; Wang et al., 2006; Zeidan et al., 2008). In fact it has recently been shown that rhinovirus activates the p38 MAPK pathway through the actions of lipid rafts and RhoA (Dumitru et al., 2006). Further investigation will be necessary to determine the role of APN and lipid rafts in the initial phase of FIPV-induced p38 MAPK activation and TNF-alpha/IL-1 beta production.

Interestingly, UV-inactivated FIPV induced prolonged p38 MAPK activation, rather than the biphasic activation induced by untreated viral particles. This suggests that FIPV may activate p38 MAPK during entry, but then suppresses p38 MAPK during the early phase of

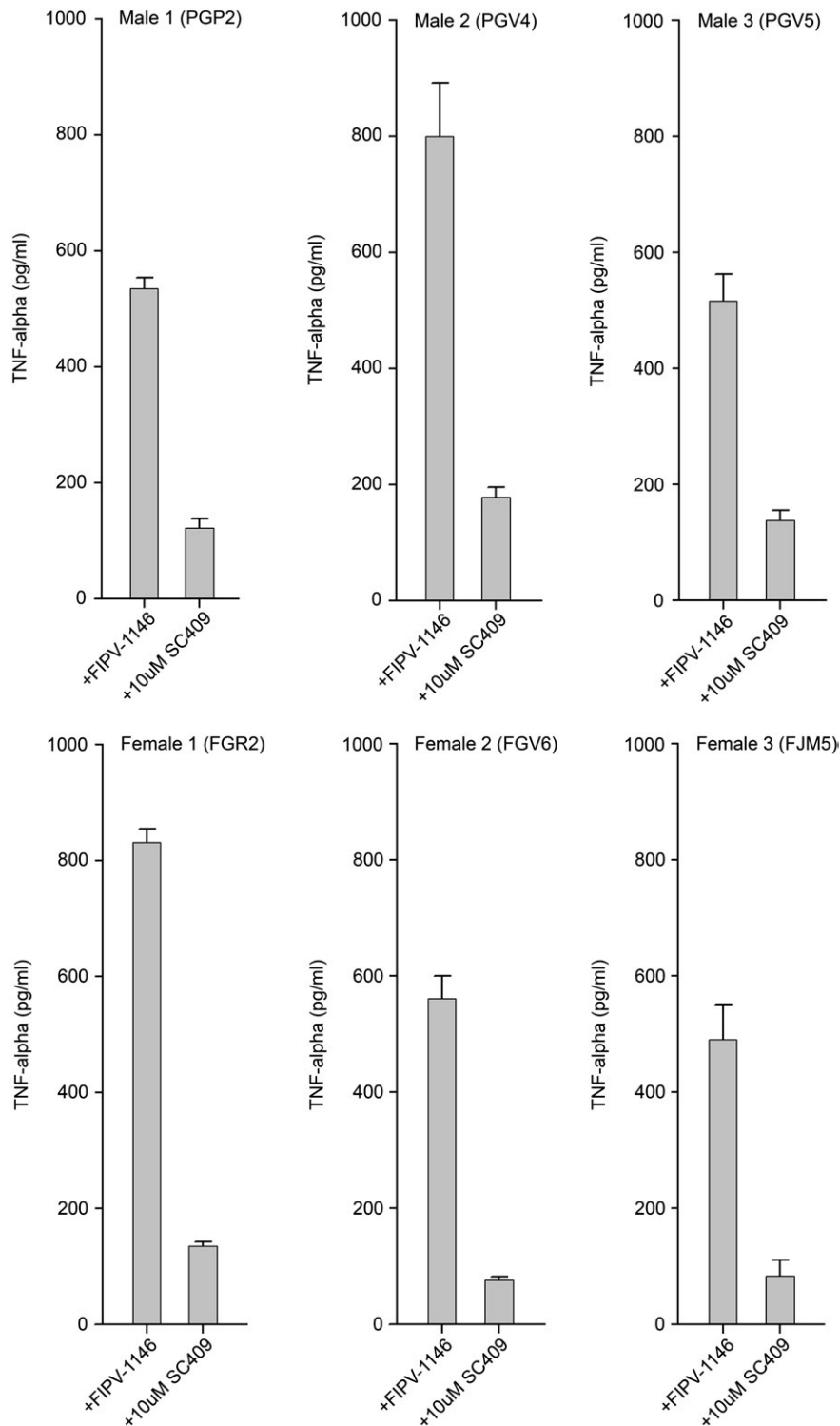


Fig. 8. FIPV-induced TNF-alpha production by PFBM cells from six SPF cats is inhibited by SC 409. PFBM cells were individually prepared from three male (07PGP2, 07PGV4, 07PGV5) and three female (07FGR2, 07FGV6, 07FJM5) SPF cats. Cells from each animal were inoculated with FIPV-1146 at an MOI of 100. 24 h p.i. supernatants were collected and TNF-alpha production was quantified by anti-TNF-alpha capture ELISA. TNF-alpha from untreated cells was below the detection limit of the assay (<10 pg/ml).

replication. The second phase of FIPV-induced p38 MAPK activation induced by untreated viral particles (6 h p.i.) may be caused by the production of pro-inflammatory cytokines. It has been shown that TNF-alpha can itself activate the p38 MAPK through signaling associated with the cytoplasmic domain of its receptors TNF receptor 1 (TNFR1)-associated death domain protein (TRADD) and TNF receptor-associated factor 2 (TRAF2) (Carpentier et al., 1998; Hsu et

al., 1995, 1996). Therefore TNF-alpha produced during the initial phase of FIPV-induced p38 MAPK activation, may be the cause of the latter phase of activation.

Pretreatment with the pyridinyl imidazole inhibitors SB 203580 and SC 409 blocked production of TNF-alpha and IL-1 beta suggesting that p38 MAPK directly regulates production of the cytokines in FIPV-infected PFBM cells. The upregulation of IL-6 production was not

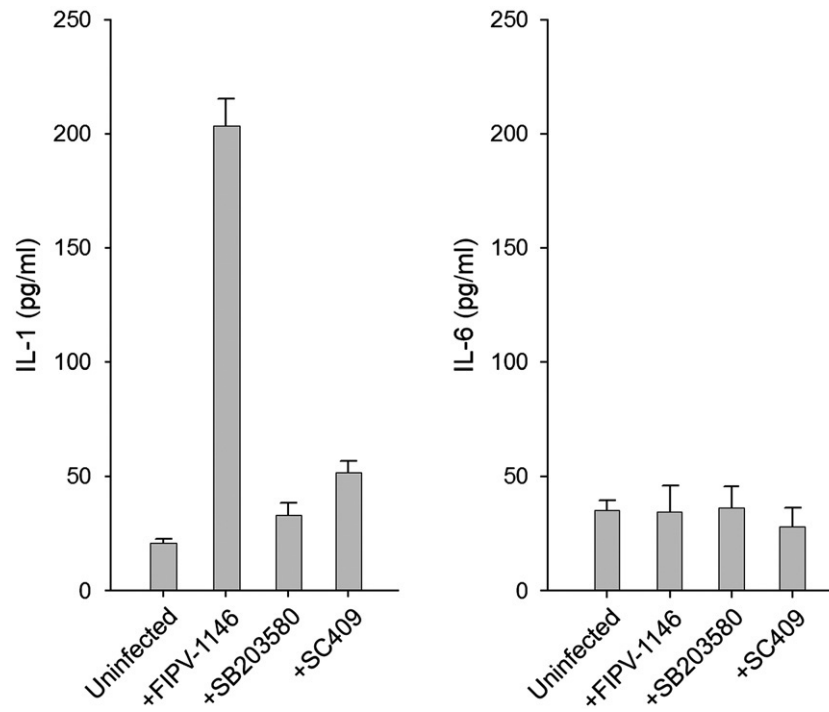


Fig. 9. Production of IL-1 beta and IL-6 by FIPV-infected PFBM cells. PFBM cells were pretreated with 10 μ M of either SB 203580 or SC 409 (or 0.1% DMSO as a control) for 2 h before inoculating with FIPV-1146 at an MOI of 100. 24 h p.i. supernatants were collected and IL-1 beta and IL-6 production was quantified by anti-IL-1 beta or anti-IL-6 capture ELISA.

observed in FIPV-infected PFBM cells, suggesting that another cell type may be responsible for its production in cats with FIP. At this time the mechanism by which p38 MAPK regulates pro-inflammatory cytokine production in FIPV-infected PFBM cells is unknown, however regulation of cytokines by MAPKs in analogous systems occurs by affecting either transcriptional regulation, translational regulation, or both (Kumar et al., 2003). For example the recently emerged severe acute respiratory syndrome coronavirus (SARS-CoV) is also known to infiltrate immune cells such as monocytes and macrophages and activate the p38 MAPK pathway (Belyavsky et al., 1998; Franks et al., 2003; Gu et al., 2005; Nicholls et al., 2003). SARS-CoV infection causes a p38 MAPK-dependent phosphorylation of downstream transcriptional regulators such as activating transcription factor 1 (ATF-1) and signal transducer and activator of transcription 3 (STAT-3), as well as translational regulators such as MAPK activate protein kinase 2 (MAPKAPK2) and the eukaryotic initiation factor 4E (eIF4E) (Mizutani, 2007; Mizutani et al., 2004a, 2004b). As seen in Fig. 2 it appears that FIPV causes increased p38 MAPK nuclear localization suggesting that the activation of transcription factors likely play a role in pro-inflammatory production in PFBM cells, however this also does not exclude a role for translational regulation. Future studies examining the role of downstream transcriptional and translational regulators in FIPV-infected PFBM cells should clarify the mechanism regulating this process.

Another aspect complicating the treatment of FIP is the diverse reactions to infection displayed by cats with the disease (Kiss et al., 2004). Our results suggest that activation of the p38 MAPK pathway and its regulation of TNF- α production is common to PFBM cells of all cats, however further sampling of animals throughout different geographic regions will be required to confirm this conclusion. Pyridinyl imidazole compounds have been shown to be efficacious therapeutic agents for blocking the mediators of chronic inflammatory diseases such as rheumatoid arthritis (Kumar et al., 2003). In fact, several p38 MAPK inhibitors have shown promise in animal models of inflammatory diseases and some have even reached human clinical trials (Kumar et al., 2003). Our results show a clear activation of p38

MAPK by FIPV during infection, and that this activation is responsible for pro-inflammatory cytokine production which is a key contributor to the pathological changes observed in cats with FIP. This raises this possibility that p38 MAPK inhibitors, alone or in conjunction with other therapies, may possess therapeutic benefits in the treatment of cats with FIP.

Materials and methods

Cells

Primary feline blood-derived mononuclear (PFBM) cells were individually purified from four male SPF cats (animal ID# 07PJO7, 07PGP2, 07PGV4, 07PGV5) and three female SPF cats (animal ID# 07FGR2, 07FGV6, 07FJM5) (Liberty Research, Waverly, NY) using a standard Ficoll-paque gradient (GE Healthcare) as specified by the manufacturer. Cells were seeded in 24-well plates with tissue culture-treated glass coverslips and allowed to attach overnight. After washing, cells were incubated in the presence of 5% CO₂ at 37 °C in RPMI-1640 media pH7.4 supplemented with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 10 μ g/ml streptomycin. The purity of PFBM preparations were routinely checked by immunofluorescence microscopy using the marker DH59B (Veterinary Medical Research & Development Inc., Pullman, WA). Crandell-Reese Feline Kidney cells were obtained from the American Type Culture Collection (ATCC) and cultured and maintained according to ATCC guidelines.

Viruses

FIPV WSU 79-1146 (FIPV-1146) was obtained from the ATCC. FIPV-DF2 was provided by Dr. Ed Dubovi (Animal Health Diagnostic Center, New York State College of Veterinary Medicine, Cornell University). Both viruses were grown by inoculating CRFK cells at a MOI of 0.01 and collecting supernatant after CPE was observed in 80% of cells which typically occurred between 48 and 72 h. Supernatant was

clarified by a low speed centrifugation step (1250× g for 10 min) and viral particles were then pelleted by centrifugation at 28,000 rpm in a SW28 rotor (Sorvall) for 60 min. Pellets were resuspended in phosphate-buffered saline (PBS). Virus titers were determined by plaque assays on CRFK cells using standard techniques. For UV-inactivation, a thin layer of viral suspension was exposed to UV light (30 W) at a distance of 10 cm for 5 min. Inactivation was verified by performing infection assays in CRFK and PFBM cells as described.

Antibodies and inhibitors

The anti-phospho-p38 MAPK (Thr180/Tyr182) (3D7) rabbit monoclonal antibody (mAb) was obtained from Cell Signaling Technologies (Danvers, MA). The anti-p38 MAPK (N-20) goat polyclonal antibody (pAb) and anti-TNF-alpha (N-19) goat pAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-FIPV nucleocapsid (N) protein mAb (17B7.1) was provided by Dr. Ed Dubovi (Animal Health Diagnostic Center, New York State College of Veterinary Medicine, Cornell University). Anti-CD127a mAb DH59B was obtained from Veterinary Medical Research and Development, Inc. (Pullman, WA). The feline TNF-alpha ELISA kit (TNF-alpha/TNFSF1A), feline IL-1 beta ELISA kit (IL-1 beta/IL-1F2), feline IL-6 ELISA kit, and associated antibodies and detection reagents were obtained from R&D Systems (Minneapolis, MN). The p38 MAPK inhibitors 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580) and 4-(3-(4-Chlorophenyl)-5-(1-methylpiperidin-4-yl)-1H-pyrazol-4-yl) pyrimidine (SC 409) were obtained from Calbiochem (San Diego, CA).

Infection assays

PFBM cells were incubated in low-serum media (1% FBS) for 12 h before inoculation with the specified virus at an MOI of 100, or pretreatment with the specified inhibitor for 2 h followed by infection. For p38 MAPK activation experiments, cells were lysed at the specified time-points in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM beta-glycerophosphate, 100 mM sodium vanadate, pH 7.4) supplemented with 1× complete protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation at 13,000 rpm in a table-top centrifuge at 4 °C for 15 min before freezing at -80 °C for later analysis. For immunofluorescence assays, cells were fixed at the specified time-points with 3% paraformaldehyde. For analysis of cytokine production, supernatant was collected 24 h post-inoculation (p.i.) before freezing at -80 °C for later analysis.

Immunofluorescence microscopy

Fixed cells were labeled with the specified antibodies as described previously (Chu et al., 2006). Cells were viewed on a Nikon Eclipse E600 fluorescence microscope, and images were captured with a Sensicam EM camera and analyzed with IPLab software.

Western blot and ELISA

SDS sample buffer was added to lysates and the reaction was heated at 95 °C for 10 min before separation using a 4–20% SDS-PAGE gel at 200 V for 2 h. Gels were electroblotted to PVDF membrane at 200 A for 2 h, blocked with 5% bovine serum albumin and probed with the specified antibody at 4 °C for 12 h. Membranes were developed using either anti-rabbit antibody (Southern Biotech, Birmingham AL) or anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz CA) linked to horseradish peroxidase and ECL substrate (Pierce, Rockford IL) and images captured using a Fujifilm LAS-3000 CCD camera. For western blot analysis of TNF-alpha production, supernatants were concentrated 50× using iCon 9 kDa molecular weight cut-off spin columns (Pierce, Rockford IL) and analyzed by western blot as described above. Western blot densitometry analysis of signal

intensity was performed using ImageJ software. For quantification of cytokine production, supernatants were processed with the specified ELISA kits (R&D Systems) using standard capture ELISA techniques as specified by the manufacturer.

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