THE ROLE OF SRC FAMILY KINASES IN THE PATHOGENESIS OF FULMINANT VIRAL HEPATITIS DUE TO MURINE HEPATITIS VIRUS STRAIN-3

by

Alice C. Wei, B.Sc., M.D.C.M

A thesis submitted in conformity with the requirements for the degree of Master of Science,

Institute of Medical Science, University of Toronto, 2000.

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ABSTRACT

Murine Hepatitis Strain-3 (MHV-3) infection of susceptible mice induces fulminant hepatitis and liver necrosis by stimulating macrophage/Kupffer cell expression of the prothrombinase, fgl-2, within hepatic sinusoids. MHV-3 induced fgl-2 activity requires activation of ERK1/2 and p38 MAPKs. To define the upstream steps of MHV-3 signaling, we examined the role of Src family tyrosine kinases in fgl-2 expression. In peritoneal exudative macrophages challenged with MHV-3, the activity of the Src kinases Hck and Lyn was increased, whereas the activity of the Src kinase Fgr was unaffected. Src inhibition prevented MHV-3 induced ERK1/2 and p38 MAPK phosphorylation, as well as fgl-2 mRNA and procoagulant activity (PCA). **Thus:** Src kinases are activated by MHV-3 and contribute to the expression of fgl-2 mRNA and PCA through the activation of ERK1/2 and p38 kinases. These studies demonstrate the crucial link of proximal protein tyrosine kinases in the signal pathways contributing to virally mediated liver disease.

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BGP, biliary glycoprotein
C-CAM, cell-cell adhesion molecule
CEA, carcinoembryonic antigen
CTL, cytotoxic T lymphocyte
EBV, Epstein-Barr virus
EGF, epidermal growth factor
ERK, extracellular signal regulated kinase
fgl-2 , fibrinogen like protein-2
G/GBV-C, hepatitis G and hepatitis GB virus C
GPI, glycophosphatidylinositol
HAV, hepatitis A virus
HBeAg, hepatitis B envelop antigen
HBsAg, hepatitis B surface antigen
HBV, hepatitis B virus
HCV, hepatitis C virus
HDV, hepatitis D virus
HE, hemagglutinin esterase
HEV, hepatitis E virus
HGV, hepatitis G virus
IFN-, interferon
IL-, interleukin
ITAM, immunoreceptor tyrosine-based activation motif
ITIM, immunoreceptor tyrosine-based inhibition motif
JNK, Jun-n-terminal kinase
LBP, lipopolysaccharide binding protein
LPS, lipopolysaccharide
MAPK, mitogen activated protein kinase
MHV-3, murine hepatitis virus strain-3
MHVR, MHV receptor
MKK, MAPK kinase
MKKK, MAPK kinase kinase
MmCGM, *mus musculus* carcinoembryonic antigen gene family member
MOI, multiplicity of infection
NCA, nonspecific cross-reactive antigen
NF-κB, nuclear factor κB
PAF, platelet activating factor
PAGE, polyacrylamide gel elecrophoresis
PBMC, peripheral blood mononuclear cell
PDGF, platelet derived growth factor
PEM, peritoneal exudative macrophage
PKC, protein kinase C
PMA, phorbol 12-myristate 13-acetate
PMN, polymorphonuclear leukocyte
PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)
PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)
RNP, ribonucleoprotein

SAPK, stress activated protein kinase
SH2, Src homology domain 2
SH3, Src homology domain 3
TF, tissue factor
TLR, toll-like receptor
TNF, tumor necrosis factor

CHAPTER 1: INTRODUCTION

I. Overview of Viral Hepatitis

Viral hepatitis remains a worldwide health problem. There are estimated to be 300-400 million carriers of hepatitis B worldwide of which 40% will succumb to complications of this disease, primarily, cirrhosis and hepatocellular malignancy (1). The majority of viral hepatitis is due to infection with one of the six unique hepatitis viruses A-G. On occasion, other viruses can cause clinical significant hepatitis (see Table I), (2), (3), (4). Clinical disease ranges from asymptomatic to fulminant fatal disease, as well as intermediate disease manifested by abnormalities in liver function tests and jaundice. Commonly, these viruses present as an acute self-limited hepatitis. Alternately, they induce a persistent, chronic hepatitis, leading eventually to cirrhosis or hepatocellular carcinoma.

Table I Causes of Viral Hepatitis

Hepatitis Viruses
Hepatitis A
Hepatitis B
Hepatitis C
Hepatitis D
Hepatitis E
Hepatitis G?
non-ABCD hepatitis
Hemorrhagic Fever Viruses
Yellow Fever Virus
Dengue Fever Virus
Rift Valley Fever Virus
Filoviruses (Marlburg, Ebola)
Other Viruses
Herpesviruses 1,2, 6
Cytomegalovirus
Epstein-Barr Virus
Adenovirus

(adapted from Acute Liver Failure, Thiele DL edited by Lee, WL and Williams, R)

I.a. The Hepatitis Viruses A-G

The majority of hepatitis seen worldwide is due to one of six identified hepatitis viruses. Hepatitis A-G (2), (3), (5). The epidemiology is highly dependent on geographic region and is constantly changing with the advent of preventative measures, including vaccination and improved sanitation strategies. The following is an overview of the major hepatitis viruses.

I.a.i. Hepatitis A

Hepatitis A virus (HAV) is a positive stranded RNA virus of the Picornaviridae family. It is transmitted exclusively by the enteric route, through direct person to person spread. HAV has high prevalence in developing countries, such as Ethiopia and Brazil, and lower prevalances in developed regions, such as North America. In developed countries, HAV is associated with childcare centers and epidemics (6). Clinically, HAV infection usually results in a mild acute hepatitis, with only a small minority of patients between 0.01 and 0.1% developing fulminant disease. Children tend to run an asymptomatic course. Increased clinical symptomatology occurs with increasing age (7). Current treatment is pre-exposure treatment with immunoglobulin and more recently with inactivated HAV vaccines (8), (9), (10).

I.a.ii. Hepatitis B

Hepatitis B virus (HBV) is the most common cause of viral hepatitis worldwide. It is a double stranded DNA virus of the Avihepadnaviridae family. Most individuals develop an acute self-limited hepatitis. Approximately 5-10% of those infected with HBV will develop chronic liver disease (2), (11), (12). Disease transmission occurs horizontally through bodily fluids or vertically, through maternal-fetal transmission. Vertical transmission is associated with very high levels of chronic hepatitis (13).

Hepatitis B virus is the major cause of fulminant viral hepatitis either alone, or in association with Hepatitis D virus infection. Certain genetic mutations of HBV have been associated with outbreaks of fulminant disease. Hepatitis B envelop antigen (HBeAg) negative mutants have been associated with epidemics of fulminant hepatitis. These virulent viruses have mutations in the precore region of the viral genome preventing synthesis of HBeAg (14), (15). HBeAg is thought to promote host immunotolerance to HBV. Thus, absence of HBeAg may predispose to an exaggerated immune response against host cells accounting for the over-exuberant hepatic damage that occurs in fulminant hepatitis (15).

Chronic HBV infection predisposes to cirrhosis and hepatocellular carcinoma. Approximately 1.5% of cirrhotics develop hepatocellular carcinoma per year. HBV related malignancy develops at a faster process than HCV related malignancy (12), (16). The recent development of a recombinant vaccine against HBV is a major step in preventing the morbidity of this viral infection.

I.a.iii. Hepatitis C

Hepatitis C virus (HCV) is a positive stranded RNA virus of the flaviviridae family. It is closely related in nucleotide and protein sequence to the recently identified Hepatitis G Virus (HGV) group. This virus is the major cause of non-A non-B viral hepatitis and transfusion related hepatitis and is primary transmitted by parenteral routes (17), (18). It is estimated to affect up to 1% of the population worldwide (17), (18). As with the other hepatitis viruses, clinical disease ranges from asymptomatic disease to fulminant disease. However, HCV is more likely than HBV to cause chronic infection (up to 50%) and is less frequently associated with fulminant disease (4). Interferon-α (IFN-α) treatment alone or in combination with ribavirin can reduce the biochemical response in a minority of patients, however its value in preventing the long term sequelae of disease, cirrhosis and hepatocellular malignancy is unknown (19), (20).

I.a.iv. Hepatitis D

Hepatitis D virus (HDV), also called the Delta Agent, is a satellite virus that causes disease only in association with HBV. This small rod shaped RNA virus has an outer layer containing Hepatitis B surface antigen (HBsAg) surrounding an inner core containing HDV RNA and an unique HDV antigen. Levels of HDV co-infection of HBV carriers is most prevalent in Southern Europe, where 17.9% of HBV carriers have anti-HDV antibodies (21). Simultaneous co-infection or super-infection with HDV often increases the severity of clinical disease. Up to 20% of HDV infection is associated with fulminant disease (4), (21), (22), (23). Interestingly, mortality due to HDV associated acute liver failure is significantly lower than mortality due to HBV infection alone (24). Simultaneous infection with both HDV and HBV leads to chronicity in 10% of patients, with rapid progression to end stage liver disease. On the other hand, most patients with established HBV that are super-infected with HDV develop chronic HDV infections. Curiously, HDV infection can occasionally result in complete clearance in HBV Ag (25),(26).

I.a.v. Hepatitis E

Hepatitis E is an RNA virus of the Calissi family and is associated with water-borne epidemics of hepatitis in developing countries with inadequate sanitation (27). In developed countries, this disease is associated with travel from endemic regions. Classic features of this disease are a predilection for young adults and a high rate of fulminant disease in pregnant women (4), (28). There is currently no specific treatment for this disease. Recombinant vaccines are currently being tested for human use.

I.a.vi. Hepatitis G

Hepatitis G and Hepatitis GB virus C (G/GBV-C) a closely related isolate, are RNA viruses of the flaviviridae family that has been recently identified (29), (30). This virus was initially identified as a transmissible agent in blood. However, several recent studies have cast doubt on the hepatotoxicity of G/GBV-C (31). Studies looking at RNA positivity show carriage rates of 1% in developed countries and up to 10-20% in developing countries (31). Thus a causative role for G/GBV-C in liver disease remains to be established.

I.b. Pathogenesis of viral hepatitis

The pathogenesis of viral hepatitis is poorly understood. These viruses are not highly virulent, nor directly cytopathic to hepatocytes. Since host factors such as age, gender and immune status are important in determining the extent of clinical disease, it is hypothesized that the immunologic response of the host plays a key role in the pathogenesis of disease. This is supported by evidence that indeed, immunologic pathways initiated by viral infection, both cellular and humoral, are the final common pathway of parenchymal destruction, and play a more important role than direct viral toxicity in hepatocyte death and the resultant liver failure.

I.b.i. Viral Factors

Directly cytotoxic factors have not been identified for the hepatitis viruses. Some serotypes are associated with more or less symptomatic disease but the extent of clinical disease is linked to modulation of the immunologic immune response. Viral factors, including mechanisms for immune evasion, result in persistence of viremia in chronically infected patients and ongoing immunologically mediated damage. Viruses use several strategies to evade the immune system, including direct interference with anti-viral immune mechanisms, escape mutation and latency. Viruses such as Epstein-Barr Virus (EBV), poxvirus and Herpes Virus

Saimiri produce cytokine-like products that interfere with the normal host response. EBV produces an IL-10 like molecule that mediates immunosuppression via its IL-10 like effects. Poxvirus on the other hand secretes an IFN-γ receptor-like molecule that antagonizes the effects of IFN-γ (32), (33).

An understanding of the pathogenesis of HBV and HCV has been hampered by the restricted host range and the difficulty propagating these viruses *in vitro*. Transgenic mice have been generated that express HBV proteins and replicate the viral genome (34), (35). Viral replication and viral protein expression is tolerated by the murine host, suggesting that HBV is not cytopathic in these systems. This further supports evidence that HBV is not directly cytopathic to hepatocytes. Thus, other factors, particularly the immune system, are primary participants in the disease process.

Other evidence also points to the primacy of the immune response in determining the extent of clinical disease. Identical innoculum of Hepatitis B injected into healthy individuals cause a spectrum of clinical outcomes, suggesting features other than viral virulence are important in the development of hepatitis (36), (37). As well, serum viremia does not correlate with the degree of liver dysfunction. Patients with fulminant HBV infection often have no serologic evidence of HBsAg, HBeAg or HBV-DNA. (15), (38), (39). Several studies have shown that in acute and chronic HBV infection, normal liver function is preserved despite high levels of viremia, with serum transaminases increasing as viremia declines (40), (41), (42). These data support the role of the host immune response as the final effector of cellular damage in clinical hepatitis.

Despite the crucial role of the host immune system, viral properties are clearly required to initiate and modulate the host response. Different HBV strains predict a more or less virulent course of disease. In HBV infection, HBeAg is thought to immunomodulate B and T cell

response to HBV by inducing immunotolerance and viral persistence. HBeAg is a secreted protein that is generated from translation of the precore and core regions of the HBV genome. It shares significant identity with HBcAg and is able to downregulate immunologic responses to HBcAg and HBeAg. Neonatal trans-placental exposure to HBeAg leads to immunotolerance of HBcAg, perhaps through elimination of HBeAg specific T cells, thus preventing destruction of HBV containing cells and leading to high levels of chronic viremia (43). Additionally, HBeAg downregulates human IFN-β production in transfected cells (44), (45). Variants of HBV which are HBeAg deficient have been epidemiologically linked to fulminant hepatitis B disease (14), (46), (47). As well, the emergence of HBeAg negative mutations is associated with flares of active hepatitis. Loss of the immunoregulatory role of HBeAg leads to 'normal' activation of the immune response, leading to an exaggerated immune response in fulminant hepatitis and an acute hepatitis flares in chronic HBV disease.

Less is known about the role of different HCV proteins and disease progression. Fulminant hepatitis due to HCV is an uncommon event and is associated with high levels of viremia, unlike HBV related fulminant disease. As yet unidentified virally specific factors may be present in HCV serotypes that cause fulminant hepatitis. In chronic HCV liver disease, HCV responds to immunologic selection pressures through escape mutation to evade the immune system. This leads to persistent low level viremia and immune mediated tissue destruction. The high rate of HCV mutation is due to its RNA template and its lack of error correction (18).

I.b.ii. Host factors

In response to infection with viral pathogens, the immune system is activated. The interaction between the viral pathogen, macrophages and lymphocytes leads to a complex series of events that result in viral clearance and associated tissue damage. T lymphocytes are activated by recognition of foreign antigens bound to self-Major Histocompatibility Complex (MHC)

molecules on the surface of professional antigen-presenting cells, such as macrophages and dendritic cells. T lymphocyte binding to the foreign antigen-self-MHC complex leads to the activation, clonal expansion and differentiation of the naïve T cells into effector cells. These activated T lymphocytes elaborate multiple cytokines which, in turn, lead to activation and potentiation of the immune cascade, leading to activation of T and B lymphocytes, endothelial cells, platelets, leukocytes and macrophages. In addition to direct lymphocyte mediated tissue damage, elaborated inflammatory cytokines such as TNF-α, IFN-γ and leukotriene B4 have been shown to mediate direct liver injury (33), (48), (49), (50).

Immunologic activation of the coagulation cascade is common in many inflammatory processes, infectious or traumatic in origin. Pro-inflammatory mediators, IL-1β and TNF-α are known to induce cellular procoagulants such as tissue factor (TF) in endothelial cells (51), (52), (53), (54), (55). Cytokine activation of endothelial cells, platelets, and TF expressing monocytes result in a net pro-coagulant milieu.

The coagulation cascade is activated by liver injury and likely represents a final common pathway of tissue destruction due to different insults. Pathologic examination of livers due to fulminant hepatitis of viral or toxic/chemical etiology in both humans and rodent models show evidence of extensive coagulative changes. Liver sections from both clinical and experimentally induced fulminant viral hepatitis show extensive sinusoidal thrombosis surrounding regions of hepatocellular necrosis (56), (57), (58), (59), (60).

The pathogenesis of fulminant viral hepatitis involves inappropriate activation of the coagulation cascade. In a murine model of fulminant viral hepatitis, infection with Murine Hepatitis Virus Strain-3 (MHV-3) leads to a strain specific hepatitis. In susceptible mice, MHV-3 infection produces a fatal hepatitis in 4-6 days. MHV-3 induces expression of fgl-2, a direct prothrombinase molecule, by macrophages/Kupffer cells. This 70 KDa procoagulant, absent in

resting cells, is induced in several systems, human and murine, and directly cleaves prothrombin to thrombin (61), (62), (63), (64) (65), (66), (67), (68). Immunohistochemical studies demonstrate the widespread deposition of fgl-2 in hepatic sinusoids, correlating with regions of hepatocellular necrosis (65), (69). Several lines of evidence support the causative role of fgl-2 in the pathogenesis of fulminant hepatitis. First, the degree of hepatocellular damage correlates with levels of fgl-2 induced procoagulant activity (PCA). Macrophages from MHV-3 susceptible mice express large amounts of fgl-2 associated PCA upon viral stimulation whereas macrophages from MHV-3 resistant mice fail to do so (61). Second, pharmacological inhibition of PCA with prostaglandin E2, which prevents PCA expression *in vitro* and *in vivo*, prevents the development of hepatitis (70), (71), (72). Third, treatment of mice with an antibody directed against fgl-2 prothrombinase prevented MHV-3 associated mortality (73). Thus, MHV-3 induction of fgl-2 prothombinase initiates the coagulation cascade through fibrin deposition, leading to sinusoidal thrombosis and ischemic hepatocellular necrosis. It is hypothesized that activation of the coagulation pathway with coagulative necrosis and hepatocellular injury is a final common pathway of tissue damage in virally induced hepatitis.

In HBV infection, the cellular immune response is responsible for viral clearance and disease pathogenesis. In the majority of patients who develop acute Hepatitis B, viral infection triggers a polyclonal and vigorous T cell response occurs, which successfully clears the virus without untoward tissue damage. In patients who develop chronic HBV liver disease, initial viral infection triggers only a weak T cell response, leading to viral persistence and smoldering T cell mediated tissue damage. In contrast, fulminant HBV disease results from an overly exuberant immune response that rapidly clears viremia but leads to massive tissue damage and hepatocellular necrosis.

In acute self-limited hepatitis in humans, HBV infection triggers a polyclonal, multi-specific T cell response with CD8+ cells directed against multiple epitopes of the HBV envelope, core and polymerase proteins. The CD4+ response is directed against epitopes of the core and precore proteins (*i.e.* HBcAg and HBeAg). Thus, both arms of the T cell response are involved in viral clearance. In contrast, patients with chronic hepatitis generate only weak cytotoxic T lymphocyte (CTL) responses to viral proteins (11), (74), (75). Thus, the extent and manifestation of liver damage that occurs in response to HBV infection is determined by the nature and amplitude of the immune response. A narrow therapeutic window exists between an appropriate immune response that is capable of clearing viremia with limited parenchymal injury. Outside this therapeutic window an inefficient immune response leads to chronic disease whereas an overly strong immune response leads to acute fulminant disease due to large scale parenchymal destruction.

Transgenic mice which expresses HBV proteins have been generated and used as a model of *in vivo* HBV infection (35), (34). Moriyama *et al* showed that adoptive transfer of CD8+ CTL to mice expressing HBsAg results in acute hepatitis. In this model, liver injury occurs in a step-wise fashion. Initially, CTL bind to HBsAg expressing hepatocytes inducing apoptosis. Also, these activated CTL recruit inflammatory cells such as PMN and lymphocytes such that, at 4-12 hours, focal regions of necrosis are seen. In mice expressing large amounts of viral antigen, an exaggerated immune response is seen with continued recruitment and activation of lymphocytes and a marked infiltration with activated macrophages leading to fulminant hepatitis, liver failure and death within 48-72 hours (11), (76), (77).

As with HBV, the immunologic response to HCV infection is the primary mediator of liver damage. In response to HCV infection, a brisk and specific CTL response against HCV core and envelope proteins is mounted (78), (79). However, the CTL response is inefficient at

viral clearance. Thus, continual HCV viremia leads to persistence of CTL mediated parenchymal damage and chronicity of hepatitis (18). A primary mechanism of HCV persistence is continual mutation of HCV to evade immune detection. This is important in ongoing injury since it promotes ongoing viremia and continued CTL associated tissue injury. Interestingly, fulminant hepatitis due to HCV is associated with high levels of viremia unlike HBV induced fulminant liver failure, suggesting that their pathogenesis differs.

Much less is known about the host responses to the other hepatitis viruses. However a common theme of viral activation of both the humoral and cellular arms of the immune system occur with significant tissue damage occurring as a result of either direct cell killing by CTL or by non-specific damage due to activation of effector cells, in particular macrophages.

I.c. Current Strategies for Viral Hepatitis

The current strategies for treating viral hepatitis includes primary prevention, therapies directed against viral pathogens, therapies directed against the immunologic response of the host as well as therapies for the failing organ. Vaccines are currently available for HAV and HBV (80), (81), (82). The recent introduction of recombinant HBV vaccines will likely have a great impact on the incidence of HBV disease induced hepatitis and hepatocellular carcinoma worldwide.

Several trials have examined treatment of chronic HBV and HCV with IFN treatment alone or in combination with ribavirin. IFN-α results in a 36-45% remission rate in selected patients with HBV. In long term follow-up studies, it appears that those who respond have a sustained response with a diminished propensity to hepatocellular carcinoma (83), (84), (85), (86). The results with HCV are less encouraging. IFN-α treatment alone results in short term improvement in more than half of the patients. However, most will relapse after the cessation of IFN treatment (87). Recent studies with combined IFN and ribavirin treatment have showed a

higher response rate with combination treatment (88), (89), (90), (91). However, long term results of combination treatment are unknown.

For HBV disease, the use of nucleoside analogs, such as adenine aribonside, acyclovir, famciclovir and 3'-thiacytidine, have been used. Lamivudine, 3'-thiacytidine, a deoxynucleoside analog and reverse transcriptase analog has been especially promising. Several trials have shown a decrease in HBV DNA in response to Lamivudine (92), (93).

The current treatment of fulminant hepatitis is supportive care with timely liver transplantation. Several different therapeutic modalities have been attempted with limited success. Strategies directed against the host immune system have been tried. In animal models of fulminant hepatitis due to MHV-3, monoclonal antibodies directed against the virally induced macrophage procoagulant molecule fgl-2 attenuated MHV-3 induced fulminant liver failure (73). In the same model, prostaglandins, particularly prostaglandin E, were protective against hepatocyte disease (94). Preliminary studies by Levy *et al* showed some survival benefit with PGE-2 treatment in humans with fulminant viral hepatitis (95), (96). However, this have not been confirmed in larger studies (97).

Other studies using immunomodulatory therapy with corticosteroids or IFN, have had conflicting results. In a double-blind randomized controlled trial, hydrocortisone was found to be unhelpful in acute liver failure (98). Interferon-α was shown in a small uncontrolled trial to ameliorate the course of aggressive viral hepatitis. However, no randomized controlled trial has shown a definitive survival benefit for IFN-α (99).

Antibodies against various cytokines have been examined in human and animal models of endotoxemia, with no clearly determined clinical benefits. It is possible that these agents may ameliorate the course of fulminant hepatitis, However, none of these agents have been examined to date.

II Signal transduction in macrophages

II.a. Overview of signaling in macrophage

Interaction between an individual cell and its environment involves communication of information from the extra, intra and intercellular domains. Signal transduction, the process by which this information is communicated, is one of the fundamental preoccupations of a cell. A complex, intricate web of signaling circuits are used to integrate signals from the environment to the nucleus of the cell, allowing the cell to adapt and respond to its environment. Some signals initiate cell activation, differentiation, division and yet others commit the cell to apoptotic death. The basic signaling circuit includes a signal-receiver, a signal-transducer and a signal amplifier. However, these signaling modules are not activated in isolation. There are tremendous, stimuli specific interaction, cross-talk and mutual regulation between different signaling networks that allow integrate signals and mediate fine control and subtlety in the ultimate cellular response that results.

Extracellular signaling is often mediated through the cell membrane by discrete cell surface receptors through various circuits, which serve to integrate the final message to the nucleus. Bacterial subunits, opsonized antigen, complement products and adjacent cells interact with macrophages by binding to specific receptors on the surface of the macrophage. For instance, CD14 receptor is a major cellular receptor for LPS whereas Fc receptor binds the constant region of antibody. Receptor binding initiates a signaling cascade which transmits and integrates cellular signals to the nucleus. These cascades are characterized by rapid and transient changes in the concentrations of intracellular second messenger molecules that trigger enzymatic or structural changes in its downstream effector enzymes or molecules. Signaling pathways include those leading to activation of protein tyrosine kinases, mitogen-activated protein kinases (MAPKs) and the cAMP-Protein Kinase C pathways, among others.

The signaling pathways that lead to macrophage activation are only beginning to be elucidated. The activation status of an individual molecule or pathway is the summation of a number of different upstream inputs and thus is highly dependent on the stimulus, cell type and concurrent environmental factors. Thus, it is not possible to say that an individual molecule or pathway is an absolute requirement for macrophage activation. It is beyond to scope of this thesis to detail the innumerable ways for macrophage signaling pathways to be stimulated. Instead, a prototypical inflammatory stimulus, lipopolysaccharide (LPS), was chosen in order to highlight the major signaling pathways operating within macrophages. This molecule was chosen because the signaling pathways of LPS are well described, thus LPS is serves as an useful illustrative example of signal activation. Also, LPS signaling involves activation of the Src family of tyrosine kinases and MAPKs in a manner similar to MHV-3, the focus of the major work of this thesis. Further, LPS, like MHV-3, can induce the expression of procoagulant molecules, Tissue Factor (TF) in the case of LPS, by macrophages. Thus, detailed knowledge of the LPS signaling pathways may provide some insights as to the signaling of MHV-3 stimulation of macrophages.

II.a.i. LPS induced signal transduction in macrophages

Macrophages are activated by bacterial products and this activation is a crucial component of the innate immune response. The prototypical bacterial product is LPS, or endotoxin, from gram negative bacterial cell walls (100), (101), (102), (103). LPS triggers macrophage activation that leads to induction and release of a multitude of macrophage derived molecules including cytokines, TNF-α, IL-1β, IL-6, Il-10 and IL-12, growth factors, CSF-1 (104) and GM-CSF (105), MIP chemokines, prostaglandins and nitric oxide (106), (107). Importantly, LPS can activate the immune coagulation system by activating tissue factor (55), (108), (109). Direct effects of LPS and autocrine responses due to LPS induced molecules lead to macrophage activation, enhanced bacteriocidal activity, and morphologic changes.

LPS is composed of four structural regions: an O-antigen polysaccharide consisting of a series of 30 to 100 repeating polysaccharides; an outer core; an inner core; and a lipid A moiety. The lipid A moiety is responsible for most of the biological activities of LPS, though constituents of the inner core also contribute to its biological effects. It is composed of a β(1-6)-linked disaccharide with diphosphorylated diglucosamine residues containing ester and amide-linked fatty acids. The inner core contains a highly conserved pattern with three 2-keto-3-deoxyoctonic acid (KDO) residues (110).

LPS forms a complex with the serum protein, LPS binding protein (LBP). In turn, this complex binds to specific cell membrane receptors, including CD14, the scavenger receptor, CD11c/CD18 integrin, an 80 Kda protein which has been recently identified as decay accelerating factor. In addition, two receptors of the Toll-like receptor (TLR), TLR-2 and TLR-4, have been implicated in CD14 dependent transmembrane signaling (111), (112), (113). At low concentrations of LPS (<10 ng/mL) the predominant LPS receptor is CD14. Transgenic mice that express CD14 are hypersensitive to LPS induced shock (114). As well, blocking antibodies against CD14 prevent LPS induced TNF-α expression, IL-6 and IL-8 expression in human monocytes and alveolar macrophages (115), (116). In a primate model of LPS induced shock, anti-CD14 antibodies not only inhibited cytokine production, it also decreased LPS induced hypotension (117).

II.a.ii. Role of Tyrosine Phosphorylation in LPS induced signaling

LPS stimulation of macrophages leads to a burst of tyrosine phosphorylation as early as 5 min (118), (119), (120), (121). Inhibiting tyrosine phosphorylation using tyrosine kinase inhibitors such as herbimycin A and genistein prevents LPS induced TNF-α, IL-6 and TF production (122), (123), (124). Additionally, inhibiting tyrosine phosphatase activity using sodium orthovanadate accentuated LPS induced production of TNF-α (122). Tyrosine

phosphorylation appears to occur by both CD14 dependent and CD14 independent routes since antibodies which prevent LPS binding to CD14 prevent tyrosine phosphorylation at low concentrations (< 10 ng/mL) but not at higher concentrations (120).

II.a.iii. Non-receptor Tyrosine Kinases

The mechanism by which CD14 initiates tyrosine phosphorylation is unknown. CD14 is a glycophosphatidylinositol-linked protein (GPI), lacking a cytoplasmic component required for signaling. Thus, the mechanism by which LPS induced CD14 dependent signaling was largely undetermined. Several recent studies have implicated the transmembrane proteins TLR-2 and TLR-4 in both CD14 dependent and independent LPS signaling. This family of proteins is characterized by an extracellular leucine rich-repeat domain and a cytoplasmic tail with significant homology to the IL-1 receptor, and without intrinsic kinase activity. Cells transfected with TLR-2 or TLR-4 mediate LPS signaling and cytokine expression signaling (111), (112). (113). Thus, it is likely that LPS forms a complex with LBP, CD14 and a TLR-2 or TLR-4 on the cell membrane and intracellular signaling occurs through the transmembrane TLR receptor. In addition, it has been suggested that some LPS signaling is mediated formation of caveolae rich in GPI proteins and non-receptor tyrosine kinases (125). For receptors that lack intrinsic tyrosine kinase activity, association with cytoplasmic tyrosine kinases including Src, Jak, Syk and Zap-70, are commonly used to facilitate signal propagation (126), (127). CD14 is associated with Lyn in human monocytes and CD14 occupancy results in activation of the Src family kinases, Hck and Fgr (128). Association of TLR-2 and TLR-4 with non-receptor tyrosine kinases is undetermined.

Src Family Kinases

Members of the Src family of tyrosine kinases, Hck, Lyn and Fgr are activated in response to LPS in macrophages and monocytes (122), (128). Overexpression of an activated mutant of Hck in the BAC1.2F5 cell line augments LPS induced TNF-α expression and conversely, inhibition of Hck expression using anti-sense oligonucleotides in the same model interferes with TNF synthesis (129). However, studies by Meng *et al* using triple knockout mice deficient in Hck, Lyn and Fgr do not support an obligatory role for Src kinases in LPS induced signaling. They showed a normal, if not slightly enhanced, production of IL-1, IL-6, and TNF-α and nitrites in these triple knockout animals. As well activation of MAPKs and NF-κB proceeds normally, suggesting a non-obligatory role for these kinases in the LPS response (130).

Another cytoplasmic tyrosine kinase Syk is also tyrosine phosphorylated, and associates with a 145 KDa protein in response to LPS stimulation. The 145 KDa protein also associates with Shc, an adapter molecule that plays a role in B and T lymphocyte antigen receptor signaling, suggesting that Syk-p145 may play are role in LPS induced signaling events (131). However, macrophages from Syk deficient mice had normal cytokine responses to LPS suggesting that Syk, like the Src kinases may be non-obligatory in LPS induced events (132).

Thus, the kinases responsible for initiating LPS induced tyrosine phosphorylation have yet to be clarified. Although several studies have shown that non-receptor tyrosine kinases are activated and required for LPS induction, knockout models of the Src family kinases and Syk, suggest that these kinases are not essential for LPS stimulated signaling events. But, a caveat to interpreting any studies performed in knockout systems is that in these experimental systems, related, and possibly redundant molecules pathologically compensate for the function of the "knocked out" genes. Thus, in the Hck, Lyn and Fgr deficient mice, it is possible that a yet

undetermined system has assumed the function of these kinases in both basal and LPS stimulated signaling events.

II.a.iv. Mitogen Activated Protein Kinases

Although the upstream pathways are yet to be delineated several of the proteins that become tyrosine phosphorylated as a result of LPS signaling are known. The major proteins that become tyrosine phosphorylated have been identified as members of the mitogen activated protein kinase family (MAPK). MAPKs are the serine/threonine kinases that are activated by dual phosphorylation on both tyrosine and threonine (reviewed in (133), (134)). MAPK activation occurs through sequential activation of a cassette-like pathway containing 3 protein kinases. The modular structure of these pathways has been well conserved in nature (see Figure 1.1). They are important signal transduction pathways integrating extracellular signals and coordinating appropriate cellular responses. Mammalian MAPKs are subdivided into 5 families: the Extracellular-signal regulated kinase, (ERK1/2); the p38 kinase; the Jun-n-terminal kinase (JNK), known alternately as the Stress Activated Protein Kinase (SAPK); the ERK3/4 kinase; and the ERK5 kinase families. Each MAPK family has distinct biologic functions. ERK1/2, p38 and JNK kinases are involved in signal transduction due to mitogenic, osmotic or stress induced stimuli, transmitting information from the cell surface to the nucleus. The ERK3/4 and the ERK5 families are less well characterized.

LPS activates the ERK1/2, p38 and JNK pathways in macrophages (118), (119), (120), (121), (135), (136). MAPKs are important in activating downstream nuclear transcription factors such as: elk-1/TCF for ERK1/2; ATF-2, MAPKAP kinase 2 and Elk-1 for p38; and c-Jun and Elk-1 for JNK. Inhibition of ERK1/2 and p38 kinases prevents LPS induced TNF, iNOS and PGE2 expression (137), (138), (139).

Conversely, activation of ERK1/2, using a chimeric version of one of its MKKKs, Raf-1, in RAW 264.7 cells led to a small amount of TNF mRNA expression suggesting that ERK1/2 activation was sufficient for TNF-α expression (140). It is unclear how LPS activates the MAPKs. Classic cytoplasmic protein tyrosine kinases are unlikely candidates for direct activation of MAPKs, given the requirement for dual phosphorylation. They may instead regulate upstream MKK or MKKK enzymes.

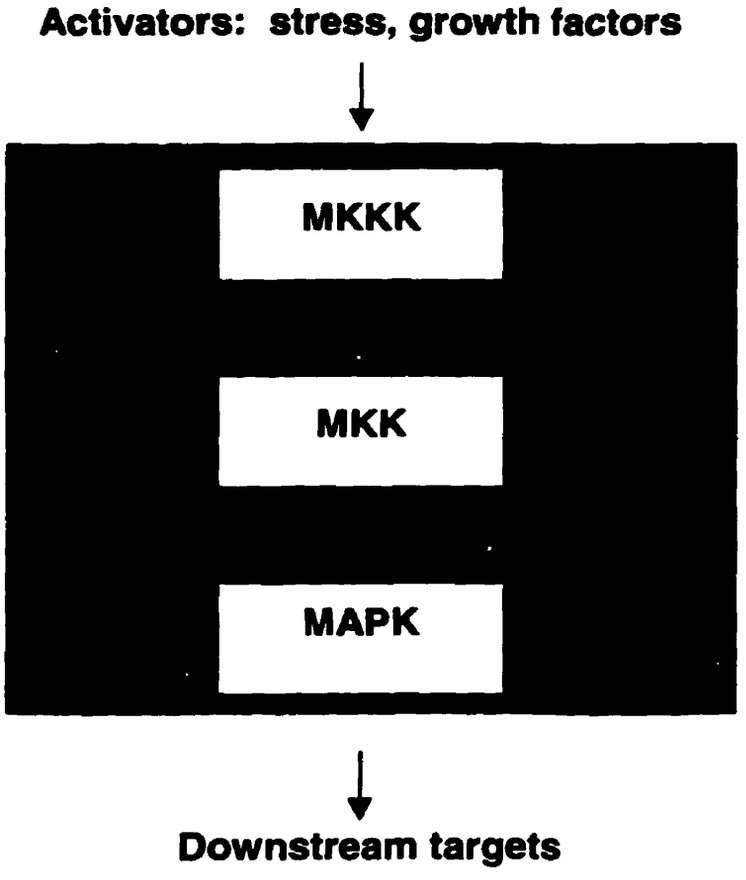

Figure 1.1. Organization of MAPK module. The MAPK module consists of three sequentially activated kinases: MKKK, MAPK kinase kinase; MKK, MAPK kinase; and MAPK.

II.a.vi. Small GTPases

Much evidence suggests a role for the small G proteins, the GTPases, particularly members of the Ras and Rho family as key upstream regulators of the MAPK pathway. Active GTP bound

Ras binds the MKKK, Raf-1, causing translocation and promoting activation of MKK1/2 and ERK1/2. Similarly, Rac and Cdc42 have been associated with the JNK MAPK cascade via its MKKK and MKK (SEK) activation. Since Rho and Cdc42 regulate cytoskeletal changes these proteins represent an integration node between nuclear and cytosketal events. In macrophage signaling, both MKKK, Raf-1, dependent and independent activation of ERK1/2 and p38 occurs. LPS induced activation of ERK1/2 and TNF is prevented by transfection of inhibitory, dominant negative Raf-1 and Ras molecules in RAW 264.7, suggesting a G protein dependent mechanism of ERK activation (141). Similar results have been found in other cell types such as HEK-293 cells, where transfection of dominant negative Ras or Raf-1 prevents ERK1/2 phosphorylation in response to G protein activation (142). As well, activation of Raf-1 in the absence of LPS leads to the induction of ERK1/2 and mimics some of the effects of LPS, including the liberation of TNF in RAW 264.7 cells. However, other LPS effects such as NF-κB activation are not activated, suggesting that LPS effects are not mediated solely though the raf-1/ERK1/2 pathway (140). On the other hand, Fcgamma receptor mediated activation of MAPK is required for cytokine expression, but is Ras and Raf-1 independent, suggesting that multiple routes to ERK activation exist (143).

II.a.vii. Protein Kinase C

The Protein Kinase C (PKC) family of serine/threonine kinases are important signal transducing agents in mammalian cells. This family of 12 kinases is also involved in signaling pathways leading to mitogenesis and prolieration, apoptosis, cytoskeletal remodeling and modulation of ion channels. Classically, PKC's are regulated by upstream lipid mediators including diacylglycerol and phosphotidylserine which bind to sequences in the regulatory domain, promoting conformational changes and exposing the catalytic site to phosphorylation and activation (reviewed in (144)). Some studies have been implicated PKCs in macrophage

activation in response to LPS (145), (146). Kovac's *et al* demonstrated that the PKC inhibitor, H7, blocked LPS induced IL-1α, IL-1β and TNF-α expression. But this association is controversial since other groups have found that LPS induced TNF-α expression is PKC independent (147). As well, other LPS responses are clearly PKC independent. However, many of these studies were done using PMA as a PKC agonist. Several isoforms of PKC are now known to be PMA insensitive, such as PKCζ, and it is possible that these atypical PKCs participate in macrophage activation events.

Despite an association of PKC activity in LPS mediated events, the precise mechanism of PKC modulation is unknown. Likely there is differential involvement of the various PKC isoforms. Several isoforms of PKC can activate Raf-1. Thus, PKC may be an upstream regulator of the MAPK pathways (148), (149). Cross-talk between pathways is also likely. It has been shown that other lipid second messengers modulate PKC activity. Products of phosphoinositide 3-kinase (PI-3K), PtdIns-3,4-P2 and PtdIns-3,4,5-P3, arachidonic acid and ceramide can modulate PKC in different systems (150). PKC also lies downstream to receptor tyrosine kinases under some conditions. Obviously, further studies are required to elucidate the nature and specifics of PKC signaling pathways in macrophages.

II.a.viii. Signal Integration, Cross talk and Mechanisms of Specificity

Cellular responses of macrophages to inflammatory stimuli such as LPS results in simultaneous and subtle changes in multiple signaling pathways. Understanding the mechanism of action has been hampered by the diverse response that occurs in different macrophage populations and concurrent environmental factors. Pathways leading to changes in GTPases, MAPK and PKC are certainly involved in LPS triggered cellular events. Extensive cross-talk between these signaling pathways both leading to positive and negative regulation occurs. This cross communication serves to integrate the signals from multiple sources. Certain pathways

such as the small GTPases and the non-receptor tyrosine kinases are found upstream of many different signals and serve a more integrative role by shuttling information from a diverse number of upstream inputs to different signaling modules. This integration of signal information is also seen at the final outcome of gene transcription regulation. Many signaling pathways lead ultimately to the regulation of transcription factors. Often, the contribution of several signaling pathways are required for the optimal expression of a given transcription factor. This represents yet another level of integration and reveals the subtle and complex control of cellular processes.

II.b. Signaling in MHV pathway

The signaling routes activated by MHV-3 infection are largely unknown. The signal transduction pathways involved in the MHV induced macrophage protein, fgl-2, are beginning to be determined. Early studies by Chung *et al* demonstrated that pretreating peritoneal exudative macrophages with PKC inhibitors, staurosporine or H7, attenuated MHV-3 induced procoagulant activity (PCA) suggesting that PKC is required for PCA. On the other hand, PKC activation with PMA did not affect PCA levels. Thus PKC activation may not be activated by MHV-3 or it may be a necessary but not sufficient requirement for MHV-3 induction of PCA. Also, it is possible that a PMA insensitive PKC isoform is activated in this process (151), (152). Further, treatment with forskolin and IBMX, agents that increase intracellular cAMP by different mechanisms of action inhibit PCA, suggesting a role for cAMP in fgl-2 downregulation (152).

More recently, MHV-3 was shown to induce rapid and transient tyrosine phosphorylation over a broad range from 35-90 KDa. Inhibition with broad spectrum tyrosine kinase inhibitors genistein and herbimycin A prevented both MHV-3 induced tyrosine phosphorylation and MHV-3 induced PCA (153). As with other inflammatory stimuli such as LPS, MHV-3 induces activation of ERK1/2 and p38 MAPK pathways. Inhibition of p38 MAPK with the selective inhibitor SB203580 prevents both fgl-2 mRNA and PCA expression whereas inhibition of

ERK1/2 with an inhibitor of its upstream MKK, PD98059 prevents fgl-2 PCA without affecting fgl-2 mRNA levels. These data suggest that p38 MAPK is involved in fgl-2 regulation at the level of transcription whereas ERK1/2 regulates fgl-2 function at a post-transcriptional level or regulates possible co-factors (154), (155).

The mechanism by which MHV-3 activates the MAPK family is unknown. The current hypothesis is one of viral binding to its specific cellular receptor and triggering the induction of a signaling cascade that leads to tyrosine phosphorylation, activation of ERK and p38, along with other yet unidentified second messengers that leads to initiation of fgl-2 gene transcription and function.

The MHV receptor, MHVR, (also known as BGP) is a transmembrane receptor of the carcinoembryonic antigen, CEA, family of the immunoglobulin superfamily. Based on analysis of CEA molecules of various origins, a number of common features have been identified (156), (157). This family of receptors is a complex group of molecules that have an immunoglobulin variable-like amino terminal domain with a variable number of immunoglobulin constant-like domains. Functionally, CEA receptors have been linked to cell-cell adhesion, signal transduction and innate immunity by acting as a bacterial and viral receptor. Some members, like MHVR, have a hydrophobic transmembrane domain and a cytoplasmic tail. Others, like CEA and nonspecific crossreacting antigen (NCA), lack a transmembrane domain and are anchored to the membrane by a glycosyl phosphatidyl inositol moiety. For molecules that are transmembrane in nature, they may have either a short 9-12 amino acid cytoplasmic tail or a longer 71-73 amino acid tail. The earliest MHVR characterized, isolated from hepatitis susceptible Balb/cJ mice, contains 4 Ig-like domains and has a short 9 amino acid cytoplasmic tail (158). However, subsequent studies have shown that multiple splice variants, including those with only 2 Ig-like domains or a long cytoplasmic tail, can bind MHV *in vitro* (159).

Analogues of MHVR, in rat and human tissues, termed CD66a or human cell-cell adhesion molecules (C-CAM), are known to contain both an Immunoreceptor Tyrosine-based Activation Motif (ITAM) and a Immunoreceptor Tyrosine-based Inhibition Motif (ITIM). These motifs are found on T and B cell receptor complexes, and Fc receptors (160), (161). When phosphorylated on tyrosine, these motifs bind protein tyrosine kinases and protein tyrosine phosphotases respectively and can initiate intracellular signaling. Antibody binding to CD66a receptors lead to tyrosine phosphorylation and binding of c-src in a colonic tumor line (162). In human neutrophils, CD66 was rapidly phosphorylated on tyrosine after fMLP, PAF or phorbol ester treatment and tyrosine kinase activity was increased, mainly due to Hck and Lyn kinases (163). Thus, it is possible that MHV-3 binds to its receptor, triggering phosphorylation of the cytoplasmic tyrosines, and leading to recruitment of protein tyrosine kinases and/or phosphatases, the initiation of intracellular signaling and ultimately, fgl-2 production.

III. Experimental Model of Fulminant Hepatitis

The pathogenesis of viral hepatitis is poorly understood. The inability to propagate human hepatitis viruses under *in vitro* conditions has hampered the understanding of viral effects on the liver. Pathologic examination of liver tissue from humans with fulminant hepatitis reveals large regions of hepatocellular necrosis, intra-hepatic fibrin deposition and sinusoidal thrombosis (59), (56), (164). These findings suggest an active role for the immune activated coagulation system as an important effector system in the pathogenesis of viral hepatitis.

Murine Hepatitis Virus Strain 3 (MHV-3) provides a useful experimental model of viral hepatitis. MHV-3 infection causes a strain specific hepatitis in mice. Fully susceptible mice (Balb/c, C57B1/6J, DBA, A/Sn, Swiss-Webster, Princeton Strain and CFW (Swiss), NZB) develop a fulminant fatal hepatitis with uniform mortality at 4-5 days. Fully resistant mice (A/J,

SJL/J) all survive viral challenge and have no biochemical evidence of liver disease (61), (165), (166), (167), (168). Semi-susceptible mice (C3H/St, C3HeB/FeJ) develop a mild acute hepatitis, which progresses to a chronic inflammatory hepatitis.

The genetic susceptibility of mice to MHV-3 induced hepatitis is due to a strain-specific macrophage activation and expression of the macrophage procoagulant molecule fgl-2. Several lines of evidence support the direct involvement of fgl-2 prothrominase in MHV-3 induced disease. First, clinical hepatitis correlates with the fgl-2 associated PCA. Thus, susceptible mice produce large amounts of PCA in response to MHV-3 infection, whereas resistant mice do not (61), (62), (63), (64). Second, fgl-2 protein expression is found in hepatic sinusoids, in the regions of hepatocellular necrosis and tissue damage (65), (69). Thirdly, neutralizing fgl-2 associated PCA with a blocking antibody prevented hepatitis, and related mortality in infected mice (73). Additionally, treatment with prostaglandin E2, which prevents PCA expression *in vitro* and *in vivo*, prevents the development of hepatitis (70), (71), (72). Collectively these data provide compelling evidence that fgl-2 expression is crucial for MHV-3 associated hepatitis.

III.a. MHV-3: Structure, infectivity and replication

Murine Hepatitis Virus Strain-3 is an RNA virus of the genus *Coronavirus*. Coronaviruses are positive stranded RNA viruses which appear as large 80-160 nm spherical particles with characteristic spike (S) proteins studding their surface and protruding 20 nm outwards from the viral envelope giving the appearance of a coronet or crown, hence the origin of the name of the genus (169), (170), (171). The genus subdivided into 3 distinct serogroups 1-3 based on antigenic specificity (see Table II). HCV 229E and TGEV are prototypical for group 1, MHV and BCV prototypical for group 2 and IBV is the sole member of Group 3.

Coronaviruses are responsible for a large number of economically important pathogens in animals and cause a spectrum of clinical disease. They characteristically have a restricted host

range and tissue specificity, infecting usually a single host species. The species and tissue specificity is largely dictated by the distribution of viral receptors. Avian infectious bronchitis virus (IBV) causes respiratory disease and nephritis in domestic fowl. Enteritis is seen with

Table II. Classification of Coronaviruses

Serogroup	Species	Designation	Clinical Syndrome
1	Porcine transmissible gastroenteritis virus	TGEV	diarrhea
	Human coronavirus 229E	HCV 229E	upper respiratory tract, enteritis
	Feline infectious peritonitis virus	FIPV	peritonitis,granulomatous inflammation
	Canine coronavirus	CCV	diarrhea
	Porcine epidemic diarrhea virus	PEDV	diarrhea
2	Murine hepatitis virus	MHV	hepatitis, encephalitis, diarrhea
	Human coronavirus OC43	HCV OC43	upper respiratory tract, enteritis
	Bovine coronavirus	BCV	diarrhea
	Porcine hemagglutinizing encephalomyelitis virus	HEV	vomiting, wasting, encephalitis
	Turkey coronavirus	TCV	enteritis
	Sialodacryadenitis virus	SDAV	adenitis
	Rabbit coronavirus	RCV	pneumonitis, rhinitis
3	Avian infectious bronchitis virus	IBV	respiratory disease

Porcine transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCV), turkey coronavirus (TCV), feline coronavirus (FCV, canine coronavirus (CCV) and porcine epidemic diarrhea virus (PEDV). In humans, human coronavirus (HCV) 0C43 and HCV 229E are upper respiratory infections responsible for the common cold. Additionally, coronaviral infections can cause acute and chronic encephalitis. A neuropathic strain of MHV-3, MHV-JHM, causes encephalitis which is used experimentally as a model of inflammatory demyelinating neurologic conditions such as multiple sclerosis (171), (172).

III.a.i.Taxonomy and structure

Murine Hepatitis Viruses cause natural disease in mice causing clinical disease of the respiratory, enteric, hepatic or neurologic disease. Strain differences in MHV account for different disease syndromes and virulence factors ranging from asymptomatic viremia, to pneumonitis, enterocolitis, hepatitis and encephalitis. MHV-JHM causes a chronic demyelinating neurologic disease. MHV-1, MHV-2, MHV-3 and MHV-A59 are primarily hepatotrophic, with MHV-3 manifesting a particularly virulent form of hepatitis causing fulminant, fatal hepatitis in susceptible mice.

Morphologically, coronaviruses are spherical, enveloped viral particles with a fringe of heavily glycosylated spike (S) proteins surrounding them. In addition, some coronaviruses, such as MHV, have an additional surface protein Hemagglutinin esterase (HE) that forms another layer of short spikes. Within the lipid bilayer of the viral envelope lie the integral membrane protein (M), the small envelope protein (E) as well as the intra-membrane components of the S and HE proteins. Within the viral particles the ribonucleoprotein (RNP) core, consisting of a huge 27-32 Kbp helix of genomic RNA associated with a nucelocapsid protein (N). Recently it has been suggested that the RNP forms in association with M proteins an internal icosohedral core (173).

The spike (S) protein is a 180-200 kDa glycoprotein that variably cleaved into 2 subunits S1 and S2 depending on the species of coronavirus and on the host cell type (174), (175). The S protein in MHV is cleaved up to 100% of the time. The S2 fragment forms the membrane spanning regions and the 'stalk' of the S protein and is held by non-covalent binding to the 'globular' S1 fragment. The S2 fragment shares more sequence homology between coronaviruses than the S1 fragment, which is essentially unique to each species. The S protein is involved in the induction of membrane fusion which may be important in both viral entry

processes or cytopathic effects on the cell wall (176), (177). As well, S protein is involved in the binding of specific viral receptors. In MHV, a 330 amino acid region at the N terminus is required for MHV binding to its receptor (178).

The membrane (M) protein is a 20-30 kDa structural protein that is lies in the phospholipid bilayer of the viral envelope. Its function remains unknown but it likely participates in viral assembly, since M proteins bind with the nucleocapsid N protein *in vitro* (179) and viral budding.

The nucleocapsid (N) protein is a 50-60 kDa highly basic protein that interacts with the viral genome to form the RNP. It has many serine residuces (7-11%) that are potential sites of phosphorylation. Phosphorylation status is thought to regulate the 'tightness' of binding between N protein and RNA (180). N protein binds RNA under specific conditions and contains a membrane binding domain that is thought to function as part of the RNA synthesis machinery, since treatment with anti-nucleocapsid antibodies prevents viral RNA synthesis of MHV (181), (182). As well, it is binds to the M protein and may regulate the incorporation of the RNP into the viral particle (179). Structurally there are 3 domains, which are separated by non-conserved linker regions. The amino and carboxy-terminal conserved domains are not well characterized. The RNA binding property is attributed to the middle conserved domain.

The envelope (E) protein is a 9.1-12.4 kDa membrane protein that is essential for viral assembly (183), (184). Although the precise mechanism it plays in viral assembly is not known, it has been postulated to be involved in viral budding, since it is localized to the perinuclear region and is membrane associated (184). However, clarification of this and additional functions are still required.

The Hemagglutinin-Esterase (HE) protein is a 65 kDa protein that is present only in some coronavirus species, including MHV, BCV, HCV-OC43, HEV and TCV. It has intrinsic esterase

function and promotes hemagglutination. The precise role of this protein is unknown but may affect virulence or pathogenicity (185).

In addition to encoding the structural proteins (S, E, HE, M, and N) non-structural proteins are encoded. The largest is the RNA polymerase gene which encompasses the nearly two thirds of the genome and encode proteins of several functions including an RNA-RNA dependent polymerase, a nucleoside triphosphate binding domain, a zinc finger nucleic acid binding domain, picornaviral 3C-like protease domain and two papain-like protease domains (186), (187). The remaining nonstructural genes, encoding proteins between 10-30 kDa are variable and of unknown function (188),(189).

The Coronavirus genome is the largest RNA genome known which encode 7-10 functional proteins in a nested structure such that all mRNAs share the same 3' terminus and extend a variable distance toward the 5'end. Thus all mRNAs are structurally polycistronic (169). An interesting feature of all coronavirus mRNAs is the presence of an identical leader sequence derived from the 5' end of the genome which is fused to the intergenic site of an individual mRNA (190), (191). The 3' terminus of the leader sequence contains a variable number of 5 base pair repeat (UCUAA) at which fuses with a homologous UCUAA-like sequence at the 5' end of the mRNA to be transcribed (192). It has been suggested that the copy numbers of the UCUAA-like sequence plays are important in transcriptional regulation.

III.a.ii MHV-3 Replication

Viral entry into a cell is mediated by specific binding of the coronavirus to a cellular receptor. MHV binds to a 110-120 kDa glycoprotein related to the CEA family of cellular adhesion molecules. After receptor binding MHV enters the host cell through endocytotosis as well as fusion with the cell membrane (193). Once within the cell, the virus uncoats and undergoes a primary translation phase, which likely involves early translation of the RNA

polymerase gene followed by transcription and translation of other viral proteins. The replication of MHV, like other coronaviruses, occurs exclusively in the host cytoplasm without requirement for the nucleus or nuclear RNA machinery as demonstrated by its ability to replicate in enucleated cells and in the presence of nuclear RNA polymerase inhibitors, actinomycin-D and α-amantin (194), (195). Inhibiting protein synthesis anytime during the viral cycle inhibits viral RNA synthesis. This suggests that continuous protein synthesis is required for this process (196), (197). Once viral protein and genomic RNA synthesis is underway, the assembly of viral particles begins. The minimal requirements for this process are the M and E proteins (198). The first event is likely the interaction of the viral genomic RNA and the N protein to form the RNP. Viral particles are assembled by interaction of the RNP with the structural proteins M and E in transitional vesicles between the rough endoplasmic reticulum and the Golgi complex. During its travel through the Golgi, assembled virions undergo further modifications, bud off the Golgi complex and exit through an exocytic pathway.

III.b. Murine model for fulminant hepatitis

III.b.i Basis of the Susceptibility and Resistance to MHV-3

The spectrum and severity of MHV-3 induced disease varies with genetic background and age of the murine host. Observed differences between resistant and susceptible mouse strains are due to differences in the immunologic response of the host strain towards the viral challenge. It has been demonstrated that resistant strains become susceptible to MHV-3 under conditions of immunodeficiency. For example, newborn A/J mice are susceptible within the first 3 weeks of life. As well, treatment with X-irradiation, anti-lymphocyte serum, T-cell deprivation or immunosuppressive drugs such as corticosteroids render these normally resistant mice susceptible to MHV-3 induced hepatitis (199), (200), (201). Humoral immunity alone does not

convey resistance since neither active nor passive immunization, with serum from immunized resistant mice, is able to prevent MHV-3 induced mortality in susceptible DBA/2 mice. The integrity of the cell immune system is required for resistance, since immunosuppressive techniques such as thymectomy render mice susceptible to MHV-3 infection (201). However, cellular immunity provided by transfer of educated thymus cells from resistant mice did not protect newborn mice from MHV-3 challenge (199).

The importance of the reticulo-endothelial system, in particular macrophages, in host resistance against MHV-3 was first demonstrated by Bang *et al* who noted that the susceptibility to MHV-3 of macrophages mirrored the severity of clinically observed hepatitis and was a heritable trait (168). This susceptibility is not due to differences in the ability of the virus to enter and replicate within macrophages, since MHV-3 has been shown to replicate within MHV-3 resistant A/J macrophages, abeit at 1-2 log lower titres than susceptible mice (60), (167).

The genetic control of resistance/susceptibility is multi-faceted and can be divided into the different levels of immune mechanisms. The initial site of host resistance is at the level of viral entry, with presence or absence of a cellular receptor for MHV-3. The second level of restriction is at the macrophage, and the third level is at the level of acquired immunity (202).

III.b.ii MHV Receptor

The first barrier to MHV-3 infection is the ability of the virus to enter host cells. The presence or absence of a cellular receptor for MHV-3 is the first determinant for susceptibility. Coronaviruses, in general, infect a limited number of host species due to their requirement for a specific cellular receptor to gain entry into the cell (203). MHV causes natural infection in mice only. The receptor for MHV was characterized by Dveksler *et al* using viral overlay blot analysis (158). They found that MHV-A59 bound specifically to a 110-120 kDa protein isolated from colonic epithelial cells and hepatocytes of susceptible mice. Resistant SJL/J mice and tissue from

human, cat, dog, monkey and hamster tissue did not bind MHV under similar conditions (203). (204) Murine Hepatitis Virus Receptor-1 (MHVR-1), was cloned and sequenced. MHVR-1 is a member of the Biliary glycoprotein (BGP) subfamily of the murine CEA family of adhesion molecules (158), (205) and is closely related to mmCGM1 (*mus musculus* carcinoembryonic antigen gene family member-1). It has 1 immunoglobulin-like variable domain (IgV), 3 immunoglobulin-like constant domains (IgC), a transmembrane region and a short cytoplasmic tail. Upon transfection of MHVR-1 into MHV resistant human and hamster cell line normally resistant cells became susceptible to MHV infection. However, the biology of MHVR-1 receptors has become increasingly complex since numerous variants of MHVR-1 have been identified. Splice variants that contain 2 immunoglobulin-like domains with or without a longer intracytoplasmic tail have been identified which can also bind MHV (159). As well, a second receptor mmCGM2 is expressed by resistant SJL/J murine tissue. Interestingly, tranfection of either MHVR-1 (mmCGM-1) or mmCGM-2 into resistant cell lines confers specificity to MHV infection suggesting additional factors are involved in determining the genetic resistance to MHV disease (159), (206).

III.b. iii. Mechanisms of Macrophage Resistance

The second level of genetic restriction is at the level of the macrophage. Macrophages are an important component of the innate host immune system, participating both as antigen presenting cells and as effector cells in the clearance of pathogens, including viruses. Within the liver, Kupffer cells form a functional barrier between blood-borne antigens and hepatocytes. Resistant A/J mice are susceptible to MHV infection until 3 weeks of age, at the time when maturation of the reticuloendothelial system occurs. As well, transfer of spleen cells from the adult to the newborn confers resistance (207). This essential role of macrophages was demonstrated by Bang *et al* who showed that selective cytolysis of host macrophages by MHV

corresponded to the strain susceptibility of mice to MHV disease and that this trait was genetically segregated in F2 and backcross generations (168). Thus, macrophages from fully susceptible mice, when infected with MHV-3 allow rampant viral replication, form large syncytial cells and are cytolysed. Cells from semi-susceptible mice show an intermediate level of macrophage cytolysis and a slower peak of viral replication whereas fully resistant mice require very high viral loads and long incubation times before cytopathic effects are seen. The resistant macrophages permit viral replication, however, with titres 1-2 logs lower than occur is susceptible mice (167). The presence of viral replication in resistant macrophages suggested that other macrophage dependent factors were involved in the observed resistance to disease.

Differences in macrophage immune activation

Several lines of evidence suggest that susceptibility to MHV-3 induced hepatitis is linked to the expression of the procoagulant molecule fgl-2 by cells of macrophage/Kupffer lineage as discussed above. In summary, induction of fgl-2 leads to activation of the coagulation cascade, sinusoidal fibrin deposition and heptocellular necrosis. Levy *et al* showed that susceptibility to MHV-3 correlates to the expression of procoagulant activity in macrophage *in vitro* (61). Thus, macrophages from susceptible Balb/c mice produced high amounts of PCA with *in vitro* infection by MHV-3, whereas macrophages from resistant A/J mice produced little PCA. Semi-susceptible C3H/St mice produced a moderate amount of PCA in response to MHV-3. MHV-3 induced PCA was shown to be due to expression of fgl-2, with no expression of tissue factor, another procoagulant molecule often induced in response to inflammatory stimuli such as LPS (62). Pretreatment with a monoclonal antibody which neutralized PCA (3D4.3) prevented *in vivo* fibrin deposition, hepatic necrosis and mortality in a dose dependent fashion, supporting the crucial role of fgl-2 activity in MHV-3 induced liver disease (73). The induction of PCA occurs early after viral infection, within 1.5 hours of infection, before the onset of viral replication (61).

Thus, the data suggests that macrophage restriction of MHV-3 is not due to inhibition of viral replication, rather due to macrophage dependent factors, such as fgl-2 associated PCA.

The relationship of PCA to liver disease parallels the findings in macrophages. In Balb/cJ mice, abnormalities in blood flow consisting of granular blood flow in both end hepatic and portal venules with associated sinusoidal microthrombi appear as early as 6 to 12 hours. Fibrin deposition can be identified at 12 to 18 hours. By 48 hours, widespread thrombosis and hepatocellular necrosis is present. In contrast, A/J mice maintain normal microcirculatory flow despite large amounts of viral titres (57), (60). Again, implicating a key role for PCA in the pathogenesis of MHV-3 induced fulminant liver failure.

The source of monocyte/macrophage PCA in response to MHV-3 is due to expression of the fgl-2. During MHV-3 infection fgl-2 protein is expressed only by Kupffer cells and hepatic endothelial cells. It is not expressed by hepatocytes (65). This protein is a 432 aa glycoprotein of approximately 70 kDa which is distinct from another well characterized monocyte/macrophage procoagulant molecule, tissue factor, at 47 kDa (62).

Fgl-2 is a fibrinogen like molecule that was first isolated in murine T lymphocytes. It directly cleaves prothrombin to thrombin, however, it does not contain any classic serine protease motifs (62). Pretreating susceptible mice with a monoclonal antibody directed against fgl-2 prevents in vitro induction of PCA in monocytic cells as well as hepatic necrosis and mortality due to MHV-3 infection (62), (73). Fgl-2 prothrombinase has been mapped to murine chromosome 5 (208). This is in keeping with earlier genetic backcross experiments, in which MHV-3 susceptibility was found to be transmitted in a 2 gene- non H2 linked fashion (209).

MHV-3 associated induction of fgl-2 involves tyrosine phosphorylation triggered by early virus-host cell interactions (*infra supra*) (153), (154), (210). Additionally, it appears that MHV-3 nucleocapsid protein is involved in fgl-2 transcriptional regulation (211).

Other differences in macrophage activation

Apart from differences in macrophage expression of fgl-2, there is evidence that the activation status of macrophages derived from susceptible Balb/cJ and resistant A/J mice differ in response to MHV-3 infection. Exudative macrophages from susceptible Balb/cJ mice produced greater amounts of IL-1, TNF-α, TGF-β, leukotrience B_4 and fgl-2 when challenged with MHV-3 than macrophages from totally resistant A/J mice despite equal responses to LPS stimulation (50). On the other hand, A/J macrophages produce five-fold higher levels of nitric oxide in response to IFN-γ treatment than Balb/cJ mice. Additionally, inhibiting NO with N-monomethyl-L-arginine results in loss of resistance to MHV-3 in this normally resistant strain (212).

III.b.iv. Mechanisms of Resistance by Acquired Immune systems

The acquired systems of immunity are also involved in the host response to MHV-3 infection. There is a limited role for humoral immunity since antibodies directed against MHV-3 do not provide protection to subsequent MHV-3 infection in susceptible mice nor does active immunization with inactivated virus provide complete protection (199), (213). As well, transplacental passage of anti-MHV antibodies provide limited, if any, protection of the newborn mouse (199). Despite these data, the integrity of the cellular immune system is required for resistance. Treatments that inhibit the cellular immune system, X-irradiation, treatment with anti-lymphocyte serum, graft vs. host reaction and T cell deprivation, render resistant A/J mice susceptible to MHV-3 infection (214). Resistance is associated with the development of a Th1 response whereas susceptibility is associated with a Th2 response (213). Further, treatment with the anti-viral agent ribavirin, which attenuates MHV-3 induced fulminant hepatitis inhibits the Th2 response of susceptible mice, while promoting the development of a Th1 response as its mechanism of resistance (215).

It has been shown that in susceptible mice, MHV-3 infection is associated with lymphoid organ involution, thymic, spleen and bone marrow with depletion of the Thy1.2 and surface IgM+ cellular depletion. These changes are not seen in resistant mice (216), (217). It is suggested that blockade of cell depletion is at the level of viral RNA polymerase activity, with resistant cells successfully blocking viral replication.

As well, direct lymphocyte participation is required for full expression of macrophage resistance factors such as fgl-2 and PCA (61), (218) however, the mechanism of its effects is not well understood.

Further, functional defects in both B and T cells characterized by cellular depletion, decreased cytokine production or muted responses to mitogenic stimuli have been observed in mice infected with MHV-3 (219), (220), (221), (222), (223). Splenic cells from mice infected with MHV-3 are transiently immunodepressed (224). MHV-3 infection causes thymic involution and lymphocyte depletion which is associated with increased pathogenicity of concomitantly infected pathogens such as *Trypanosoma cruzi* (225). As well, *in vitro*, MHV-3 infected lymphocytes show a diminished proliferative response in mixed lymphocyte cultures.

CHAPTER 2: HYPOTHESIS

The preceding discussion highlights the role of host immunity in determining the susceptibility to MHV-3 induced viral hepatitis. Activation of the coagulation cascade by induction of fgl-2 prothrombinase activity appears to be paramount in the pathophysiology of MHV-3 induced hepatitis. Fgl-2 cleaves prothrombin to thrombin leading to activation of the coagulation cascade, inducing fibrin deposition and thrombus formation in the hepatic sinusoids, leading to ischemic hepatocellular necrosis. The early signaling events of fgl-2 activation require tyrosine phosphorylation and the activation of ERK and p38 MAPK. The Src family of tyrosine kinases are known upstream regulators of ERK and p38 MAPK. Further, Src kinases associate with analogs of the MHV-3 receptor. Thus, we hypothesized that members of the Src family of tyrosine kinases are components of the MHV-3 induced signaling pathway and that their tyrosine kinase activity is required for fgl-2 functional activity.

CHAPTER 3: THE ROLE OF SRC FAMILY KINASES IN MURINE HEPATITIS VIRUS STRAIN-3 INDUCED FGL-2 EXPRESSION

I. Introduction

The development of fulminant viral hepatitis is associated with a high mortality rate despite the availability of liver transplantation to such patients. In an experimental model of viral hepatitis, Murine Hepatitis Virus Strain 3 (MHV-3), a single stranded, positive polarity RNA coronavirus, causes strain-specific hepatitis in susceptible mice. Susceptible strains develop a fulminant hepatitis with uniform fatality by 5-6 days, while fully resistant mice show no evidence of liver disease following infection (61), (218), (226), (227). The development of fulminant hepatitis is linked to the expression of a cell associated protein, fgl-2, characterized by its direct prothrombinase activity, leading to activation of the coagulation cascade and fibrin formation. Histologic examination of mice succumbing to MHV-3 induced hepatitis, reveals extensive sinusoidal fibrin deposition and ischemic liver necrosis, consistent with a role for this molecule in this disease (60).

The combined evidence now clearly implicates a central role for fgl-2 in the pathogenesis of MHV-3 induced liver failure. First, MHV-3 induced procoagulant activity (PCA) and upregulation of fgl-2 expression in peritoneal exudative macrophages (PEM) parallels the resistance/susceptibility to infection and clinical severity of hepatitis (50), (61), (168), (200). Second, fgl-2 expression following MHV-3 infection is primarily in the liver, and correlates with sinusoidal fibrin deposition and hepatocyte injury. Finally, strategies directed at neutralizing the molecule prevent hepatocellular necrosis in susceptible mice. For example, pretreatment of mice with a monoclonal antibody, 3D4.3. directed against MHV-3 induced PCA, prevents hepatic fibrin deposition, hepatitis and mortality (73). It is of particular interest that the human analogue of fgl-2 has been identified (67), (228). Thus, this gene product may participate in the pathogenesis of fulminant viral hepatitis in humans.

Recent studies have begun to elucidate the cellular signaling pathways leading to MHV-3 induced fgl-2 expression in peritoneal macrophages. Specifically, MHV-3 was shown to increase cellular phosphotyrosine accumulation. Inhibition of tyrosine phosphorylation with the broad spectrum tyrosine kinase inhibitor genistein, prevented fgl-2 mRNA and protein upregulation and blocked functional procoagulant activity (PCA) (153). In recent studies, we have reported that members of the MAPK family, ERK1/2 and p38, are phosphorylated and activated by MHV-3 infection. Selective inhibition of p38 and ERK causes transcriptional and post-transcriptional blockade of fgl-2, respectively (210).

Some of the initial binding characteristics of MHV-3 following infection are known. MHV-3 binds to a receptor, known as MHVR, which is a cell -cell adhesion molecule (C-CAM), in the CEA adhesion molecule family (158), (159), (229), (230), (231),(232). While the biological role of MHVR has not been fully defined, C-CAM molecules are able to function as signal-regulatory proteins. C-CAM splice variants with long cytoplasmic tails are known to contain two tyrosine residues that contribute to a modified immunoreceptor tyrosine-based activation motif (ITAM). Additionally, the membrane proximal tyrosine contributes to an immunoreceptor tyrosine based inhibition motif (ITIM). These motifs, when tyrosine phosphorylated are known to bind protein tyrosine kinases and protein tyrosine phosphatases, respectively (156). Although the classic MHVR has a short 9 amino acid cytoplasmic tail, splice variants with longer cytoplasmic tails have been described and may function as MHV-3 receptors (159).

In this regard, CD66, the human analogue of MHVR has been shown to be associated with c-Src in co-immunoprecipitiation studies (162). Src is the prototypical member of the Src family kinases, a group of 9 related non-receptor bound tyrosine kinases which contain a catalytic domain, an SH2 domain, and an SH3 domain (233). Src family kinases are known to

associate with transmembrane receptors such as PDGF and EGF receptors (234) and are involved in signaling events that result in cell cycle progression. In the immune system, Src kinases are involved in the signaling pathways that result in the activation of T and B cell receptors as well as signaling pathways leading to the induction of cytokines such as IL-1 and TNF. Based on these considerations, we postulated that Src family kinases might be involved in signaling pathways leading to the expression of fgl-2 prothrombinase following MHV-3 infection. In the present studies, we examined the role of Src family kinases in MHV-3 induced signaling and their potential role as upstream regulators of ERK1/2 and p38 MAPKs. We found that the Src family kinases, Hck and Lyn, were activated in response to MHV-3 infection, while Fgr kinase was unaffected. Pharmacologic inhibition of Src with the Src selective inhibitor PP1 markedly reduced the expression of fgl-2 prothrombinase and inhibited p38 MAPK and ERK1/2 phosphorylation. These data implicate members of the Src family kinases as being involved in the upstream signaling following MHV-3 infection, ultimately leading to induction of fgl-2 prothrombinase expression.

II. Methods

Animals: Pathogen-free female Balb/c mice aged 7-8 weeks were purchased from Charles Rives Laboratories (Constante, Quebec, Canada) and housed in the animal facility at The Toronto General Hospital. They were acclimatized for 1 week prior to use and fed animal chow and water *ad libitum*. All protocols were approved by the institutional committee for animal care.

Reagents: Endotoxin-free RPMI and Hanks Balanced Salt Solution (HBSS) were purchased from Life Technologies (Burlington, Ontario, Canada); Fetal calf serum from Hyclone (Toronto, Canada); and L-glutamine from Sigma Laboratories (St Louis, Missouri, USA). Brewer's thioglycolate was purchased from Difco Laboratories (Detroit, Michigan, USA) and prepared as a 4.5% solution according to the manufacturer's instructions. The selective Src

inhibitor, PP1 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), was purchased from Calbiochem (San Diego, California, USA) and prepared in DMSO as a 10 mM stock solution, which was further diluted in medium immediately before use (During the course of these experiments, PP1 became unavailable and was replaced with PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) which differs from PP1 in a chlorophenyl group that does not affect its ability to inhibit Src kinases). Throughout this thesis the term PP1 will be used. Lipopolysaccaride (LPS) from *E. coli* 0111:B4 used for control purposes, was purchased from Difco (Detroit, Michigan, USA) and prepared as a 1 mg/ml stock solution.

Virus: MHV-3 was purified, passaged and titred as previously described (153). In brief, virus was plaque purified on DBT cells and grown in 17 CL1 cells to a concentration of 3 X 10^7 PFU/ml. Viral titres were obtained by standard plaque assay on monolayers of murine L2 cells, as previously described (61). In addition, the effect of PP1 (10 μM) on MHV-3 viability was determined using peritoneal exudative macrophages in place of 17 CL1 cells for viral culture. The standard L2 plaque assay for determining viral titres.

Macrophage Isolation: Peritoneal exudative macrophages from MHV3-susceptible Balb/c mice were harvested by lavage with 10 mL of sterile, ice-cold HBSS, 4 days following peritoneal injection of thioglycolate. Macrophages were washed twice in cold HBSS and resuspended in RPMI supplemented with 2% fetal calf serum and L-glutamine to a concentration of 1-10 X 10^6/mL. This procedure consistently yields 80-90% macrophage population by Wright's Stain with viability > 95% by trypan blue exclusion (108).

Cell Activation: Macrophages were incubated in the presence or absence of MHV-3 with a multiplicity of infection (MOI) ranging from 1 to 5, for various times at 37°C in 5% CO_2. For some experiments, cells were pretreated for 15 min prior to stimulation with the selective Src

family kinase inhibitor PP1 (1-10 uM). Reactions were stopped by placing cells on ice and cells were lysed by the appropriate method.

Procoagulant Activity (PCA): Macrophage were pelleted after stimulation, freeze-thawed and assayed for their ability to shorten the spontaneous clotting time of recalcified, platelet-poor human plasma using a one-step clotting assay as previously described (210), (153). Briefly, 80 uL of PEM cell lysates were added to 80 uL of citrated normal human plasma. To initiate clotting, 80 uL of $CaCl_2$ (25 mM) was added and the reaction was gently agitated at 37°C until fibrin clot formation occurred. The time to clot formation was recorded and quantitated by converting clotting times to milliunits of PCA by comparison to known concentrations of thromboplastin, American Diagnostica (Montreal, Quebec, Canada). Although MHV-3 induced PCA has been shown to be due to the prothrombinase fgl-2, the dilution curve for the clotting reaction for lysed cells after MHV-3 treatment was linear and parallel to the thromboplastin standard curve (210), (153), (62), (64).

Western Blotting: At various times following stimulation, cells were pelleted and lysed in boiling Laemmli buffer. Whole cell lysates from 2.5 X 10^5 cells were separated on 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane, Millipore (Nepean, Ontario, Canada). Blots were then probed with monoclonal anti-phospho-tyrosine 4G10, Upstate Biochemical Inc (Lake Placid, New York, USA), polyclonal anti-phospho-p38 (New England Biolabs, USA), polyclonal anti-phospho-ERK1/2, New England Biolabs (Mississauga, Ontario, Canada), polyclonal anti-Hck, monoclonal anti-Lyn and anti-Fgr antibodies, Santa Cruz (Santa Cruz, California, USA). The blots were then incubated with the appropriate HRP labeled secondary antibody and visualized with an ECL based system, Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canda) as previously described (155).

Immunoprecipitation and Kinase Assays: At various times after stimulation, 5-7.5 X 10^6 cells were pelleted and lysed in cold lysis buffer (1% Triton-X 100, 150 mM NaCl, 10 mM Tris HCL (7.4), 2 mM sodium orthovanadate, 50 mM NaF, 5 mM EDTA, 10 ug/mL leupeptin, 10 ug/mL aprotinin and 1 mM PMSF). Post-nuclear extracts were collected and precleared with Protein-G Sepharose, Pharmacia Biotech (Mississauga, Ontario, Canada) for 1 hour at 4°C and immunocomplexed with either anti-Hck, anti-Lyn, anti-Fgr, Santa Cruz (Santa Cruz, California, USA), or anti-Pyk2 antibodies (Transduction Labs) by incubating for 1.5 hr at 4 °C. Protein-G Sepharose beads were added and the lysates were further incubated for a 1 hr at 4°C. The complexes were washed 5 times with ice cold PBS. For immunoprecipation studies, proteins were liberated by adding Laemmli buffer. Proteins were then separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane, Millipore (Nepean, Ontario, Canada) and analyzed by Western Blotting.

For the *in vitro* kinase activity, complexes were washed twice with ice cold kinase assay buffer, 20 mM HEPES (pH 7.4), 10 mM $MnCl_2$, 250 μM Na_3VO_4 and 1 μM [γ^{32}-P]ATP, Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canda) and then incubated for 10 min at 30°C with 2 μg dialyzed acid-treated enolase, Boehringer-Mannheim(Laval, Quebec, Canada). Reactions were stopped with boiling Laemmli containing 0.1 M DTT and the samples were boiled for 5 min. Proteins were resolved on 12.5% SDS-PAGE and autoradiograms were taken.

Northern Blotting: 10 X 10^6 PEM were pelleted after stimulation and total RNA was extracted by the single step guanidinium-isothiocyanate method of Chomcynski and Sacchi (235). Total RNA was denatured, resolved on a 1.2% formaldehyde-agarose gel and transferred to nylon membrane. Hybridization was carried out using a 1.3 Kb ^{32}P-labeled, random-primed cDNA for murine fgl2 prothrombinase (64) and analyzed by auto-radiogram. The membrane was

then stripped and reprobed with a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure comparable loading.

Statistical Analysis: Data were expressed as mean ± standard error of the mean for n experiments. One way Analysis of Variance (ANOVA) with *post hoc* Tukey analysis. Statistical significance was defined as an alpha level < 0.05.

III. Results

MHV3 induction of Src Family Kinases

The major Src family kinases present in murine macrophages are Hck, Fgr and Lyn (130). To determine whether these kinases were activated following exposure to MHV-3, *in vitro* kinase assays were performed using the exogenous Src substrate enolase. PEM were infected with MHV-3 for various times. The cells were then lysed and post nuclear extracts were immunoprecipitated with antibodies directed against various Src family kinases. These immune complexes were then incubated with acid treated enolase in an *in vitro* kinase assay. As shown in Figure 3.1, MHV-3 infection (MOI 5) caused rapid activation of Hck, peaking at 10 min. Lyn activity was also increased by MHV-3 stimulation over a similar time course (Figure 3.2). In contrast, the activity of Fgr was not affected by MHV-3 stimulation (see Figure 3.3). Thus, MHV-3 differentially effects the various Src family kinases present in macrophages, activating Hck and Lyn without an effect on Fgr.

The substrate preference of the individual Src family kinases may differ. Thus to assess whether the unresponsiveness of Fgr might be attributed to a limited affinity towards enolase, we used the synthetic polypeptide KVEKIGEGTYGVVYK, corresponding to amino acids 6-20 of $p34^{cdc2}$, that has been shown to be a preferred and selective Src family substrate (236). MHV-3 treatment failed to activate Fgr activity with this substrate as well (data not shown).

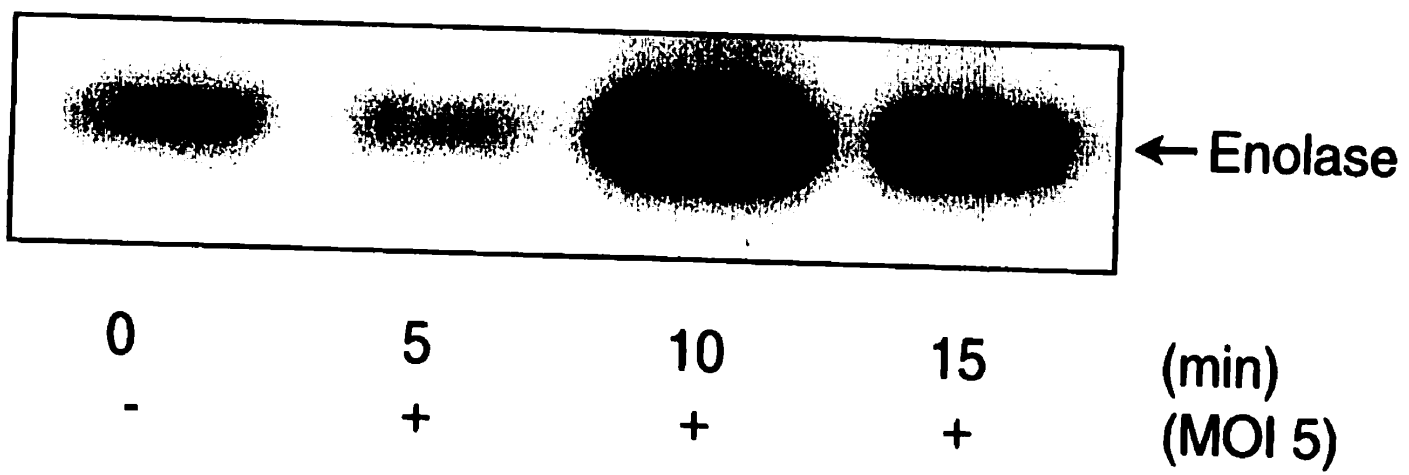

Figure 3.1. MHV-3 activates Hck kinase. PEM (7.5×10^6) were infected with MHV-3 (MOI 5) for various times at 37°C/5%CO_2. Cells were pelleted and lysed with ice cold 1% Triton-X lysis buffer. Cytoplasmic proteins were immunoprecipitated with anti-Hck, separated by SDS-PAGE, and assayed for their ability to phosphorylate the substrate enolase. Each autoradiogram is representative of at least three independent experiments with similar results. Hck activity is stimulated by MHV-3, with maximum stimulation at 10 min and fading thereafter.

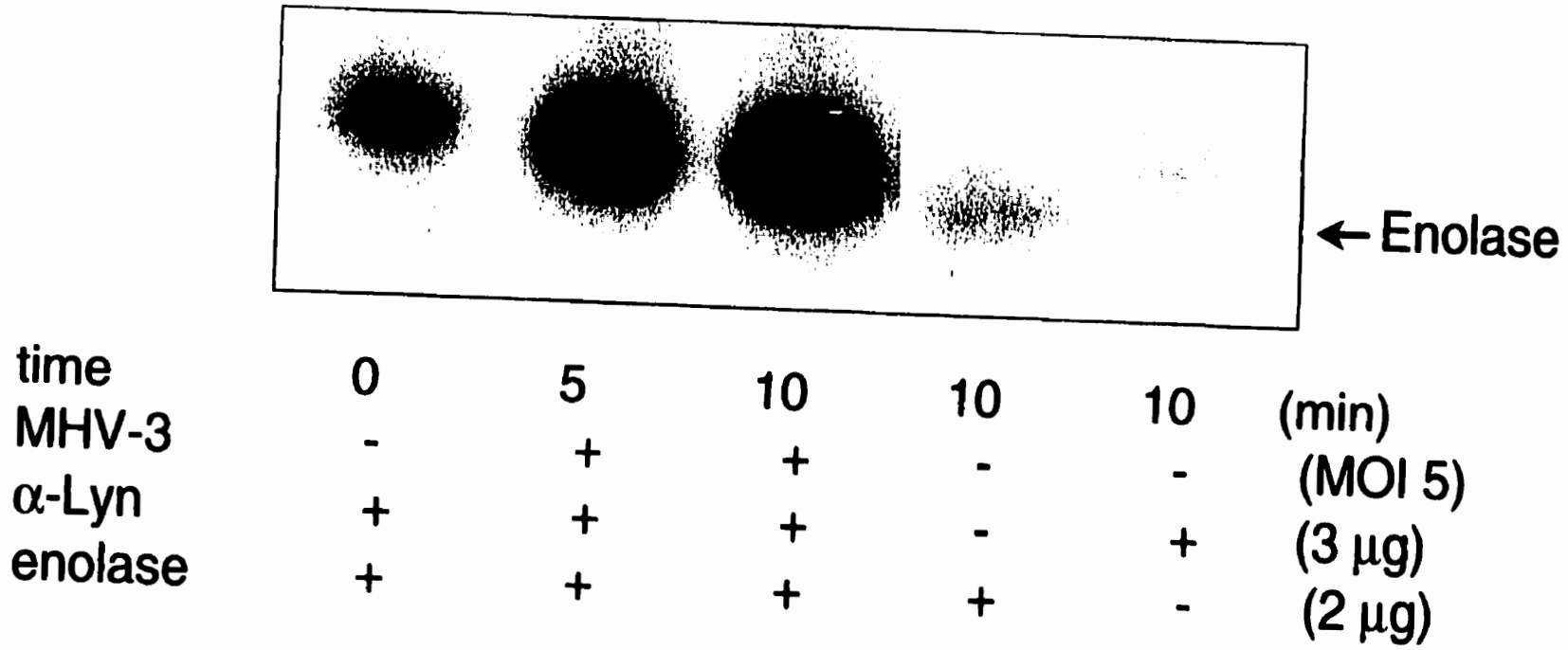

Figure 3.2. MHV-3 activates Lyn kinase. PEM (7.5×10^6) were infected with MHV-3 (MOI 5) for various times at 37°C/5%CO_2. Cells were pelleted and lysed with ice cold 1% Triton-X lysis buffer. Cytoplasmic proteins were immunoprecipitated with anti-Lyn antibody, separated by SDS-PAGE, and assayed for their ability to phosphorylate the substrate enolase. Each autoradiogram is representative of at least three independent experiments with similar results. Lyn activity is rapidly stimulated by MHV-3, peaking at 10 min. Note that the rightmost lanes are technical controls, showing macrophage in the absence of anti-Lyn antibody and enolase, respectively.

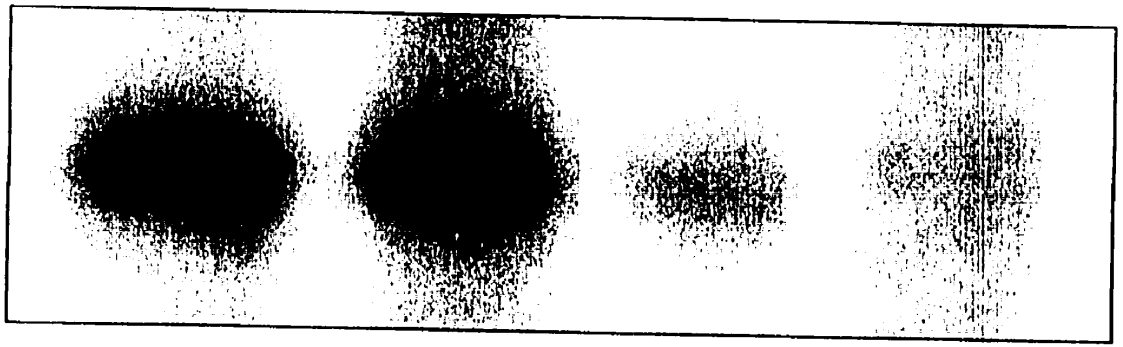

Figure 3.3. MHV-3 has no effect on Fgr kinase. PEM (7.5×10^6) were infected with MHV-3 (MOI 5) for various times at 37°C/5%CO_2. Cells were pelleted and lysed with ice cold 1% Triton-X lysis buffer. Cytoplasmic proteins were immunoprecipitated with anti-Fgr antibodies, separated by SDS-PAGE, and assayed for their ability to phosphorylate the substrate enolase. Each autoradiogram is representative of at least three independent experiments with similar results. In contrast to Hck and Lyn kinase, Fgr kinase activity is not increased over constituitive levels by MHV-3 infection. Again, the rightmost lanes are technical control lanes, showing cytoplasmic proteins in the absence of anti-Fgr antibody and enolase, respectively.

MHV-3 activates Pyk2 which is prevented by Src inhibition

To assess the physiologic relevance of Hck and Lyn activation by MHV-3. a known substrate of the Src family kinases, Pyk2, was assessed for phosphorylation in response to MHV-3 stimulation (237, 238). In response to MHV-3 stimulation (MOI 5), Pyk2 was phosphorylated on tyrosine 10 and 15 min (Figure 3.4).

Src inhibition with the highly selective Src kinase family inhibitor PP1 (10 μM) abrogated MHV-3 induced tyrosine phosphorylation of Pyk2, confirming that Pyk2 phosphorylation was a consequence of MHV-3 activation of Src kinases (Figure 3.4).

Src inhibition modulates fgl-2 *mRNA levels*

Having demonstrated activation of Src kinases by MHV-3 infection, we evaluated their role in fgl-2 expression. Northern blot analysis was used to examine steady state fgl-2 mRNA levels in control or PP1 treated cells. While fgl-2 is not constituitively expressed in unstimulated PEM, MHV-3 infection induced fgl-2 mRNA expression, increasing up to 6 hours and then gradually declining. Src inhibition with PP1 reduced fgl-2 mRNA at all time points (see Figure 3.5).

Effect of Src inhibition on MHV-3 induced Procoagulant activity

Consistent with its ability to induce the prothrombinase fgl-2, PEM infected with MHV-3 at an MOI of 1 showed a significant increase in PCA at 6 hours compared to untreated PEM (Figure 3.6). To assess the potential involvement of the Src family in the virus induced signaling, cells were left untreated or were treated with various concentrations of the highly selective Src inhibitor PP1 and challenged with MHV-3 (MOI 1). At six hours PP1 significantly inhibited MHV-3 induced PCA at concentrations of 1 uM or greater ($p < 0.05$ compared MHV-3 alone).

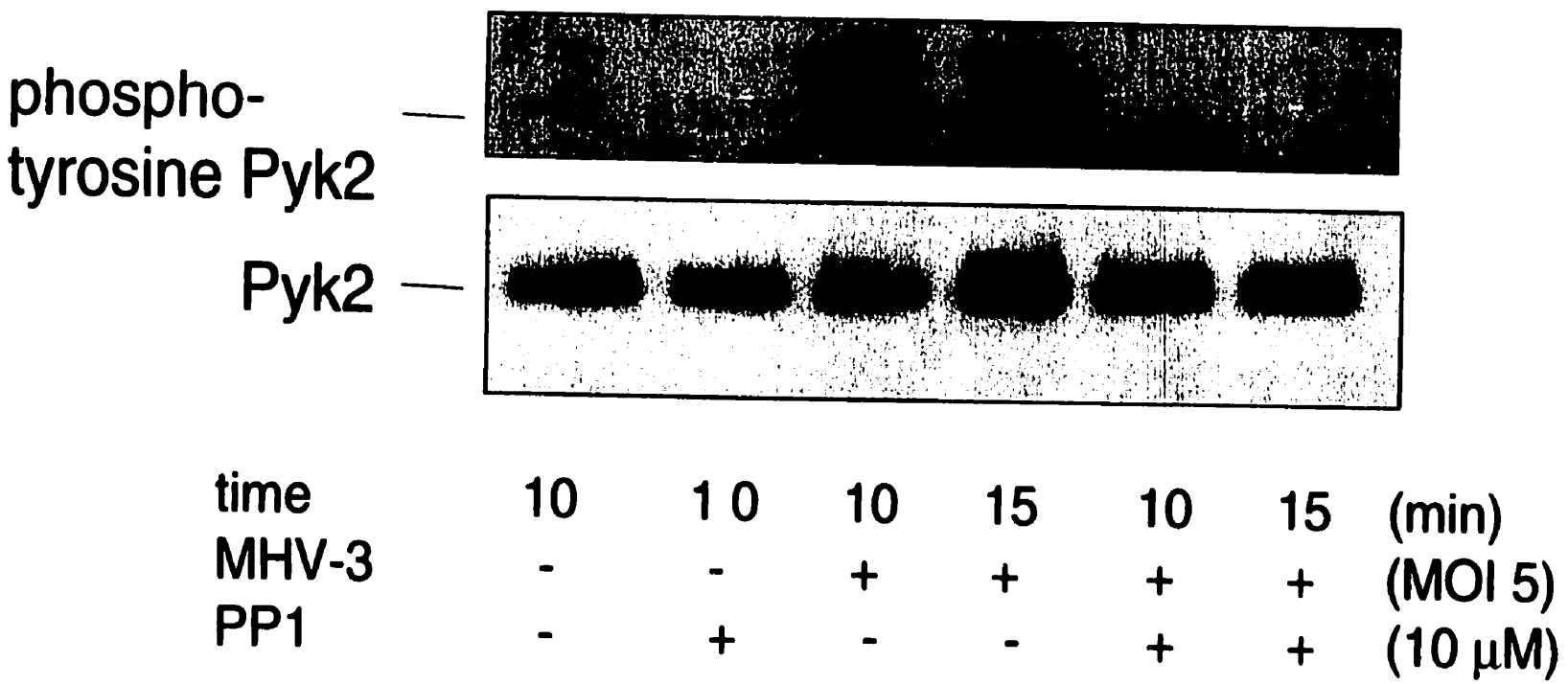

Figure 3.4. MHV-3 leads to Pyk2 phosphorylation on tyrosine which is prevented by PP1. PEM (7.5×10^6) were infected with MHV-3 (MOI 5) for 10 to 15 min at 37°C/5%CO_2. Cells were pelleted and lysed with ice cold 1% Triton-X lysis buffer. Cytoplasmic Pyk2 was immunoprecipitated and proteins were separated with SDS-PAGE and visualized by Western Blotting with anti-phospho-tyrosine antibodies. Pyk2 is not tyrosine phosphorylated in control cells or with PP1 (10 μM) treatment alone. With MHV-3 infection (MOI 5), Pyk phosphorylation occurs at 10 and 15 min. Pretreatment with PP1 prevents MHV-3 induced Pyk2 phosphorylation. The additional band in the top panel likely represents a co-immunoprecipitated protein that is itself tyrosine phosphorylated.

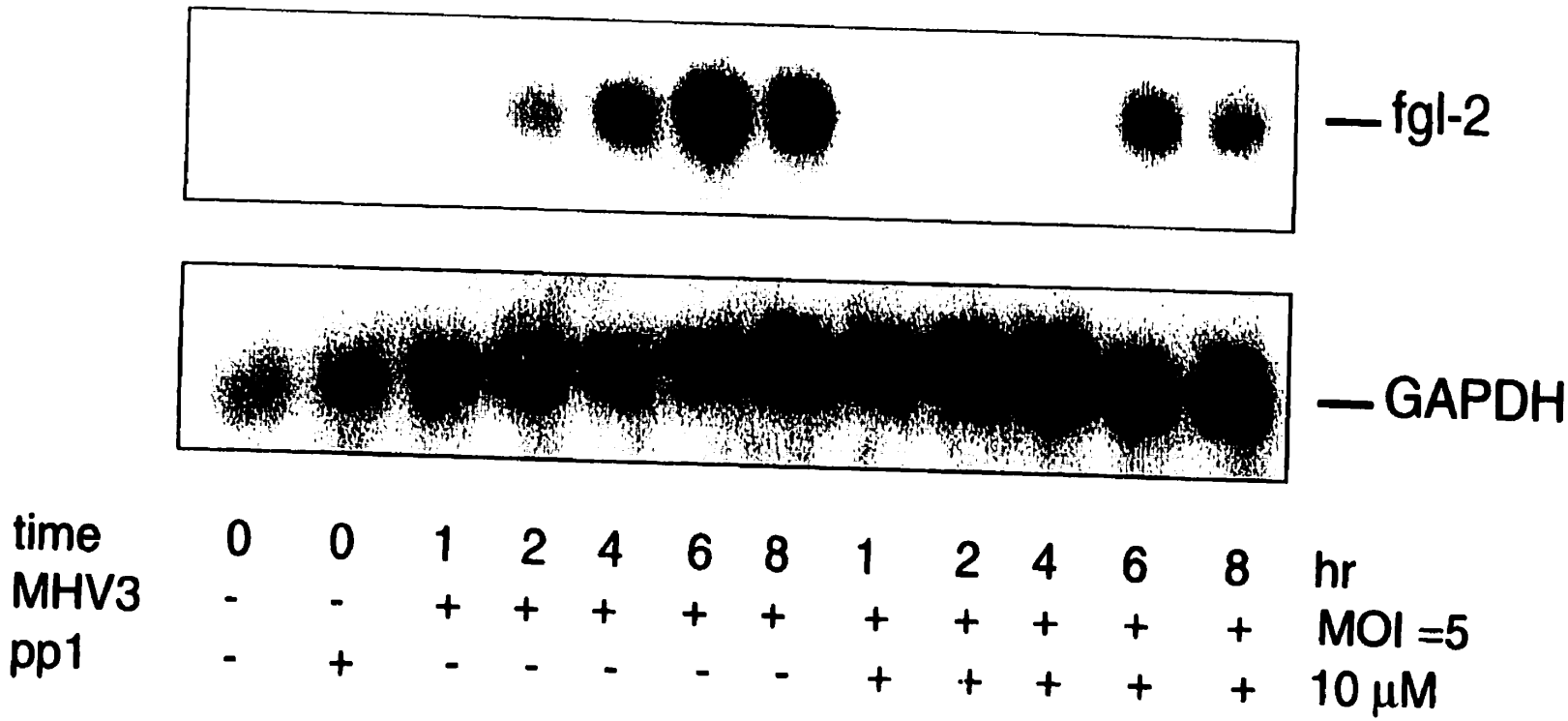

Figure 3.5. MHV-3 induced fgl-2 mRNA expression is inhibited by PP1. PEM ($10X10^6$) were infected with MHV-3 (MOI 1) and incubated for various times at 37°C/5%CO_2 in the presence or absence of PP1 (10 μM, added 15 min prior to viral stimulation). RNA was extracted and Northern blot analysis was performed with cDNA for fgl-2. The blot was then stripped and reprobed with the housekeeping gene, GAPDH, to ensure comparable RNA loading. Src inhibition by PP1 markedly inhibits fgl-2 mRNA expression at all time points. The blot is representative of at least three independent experiments with similar results.

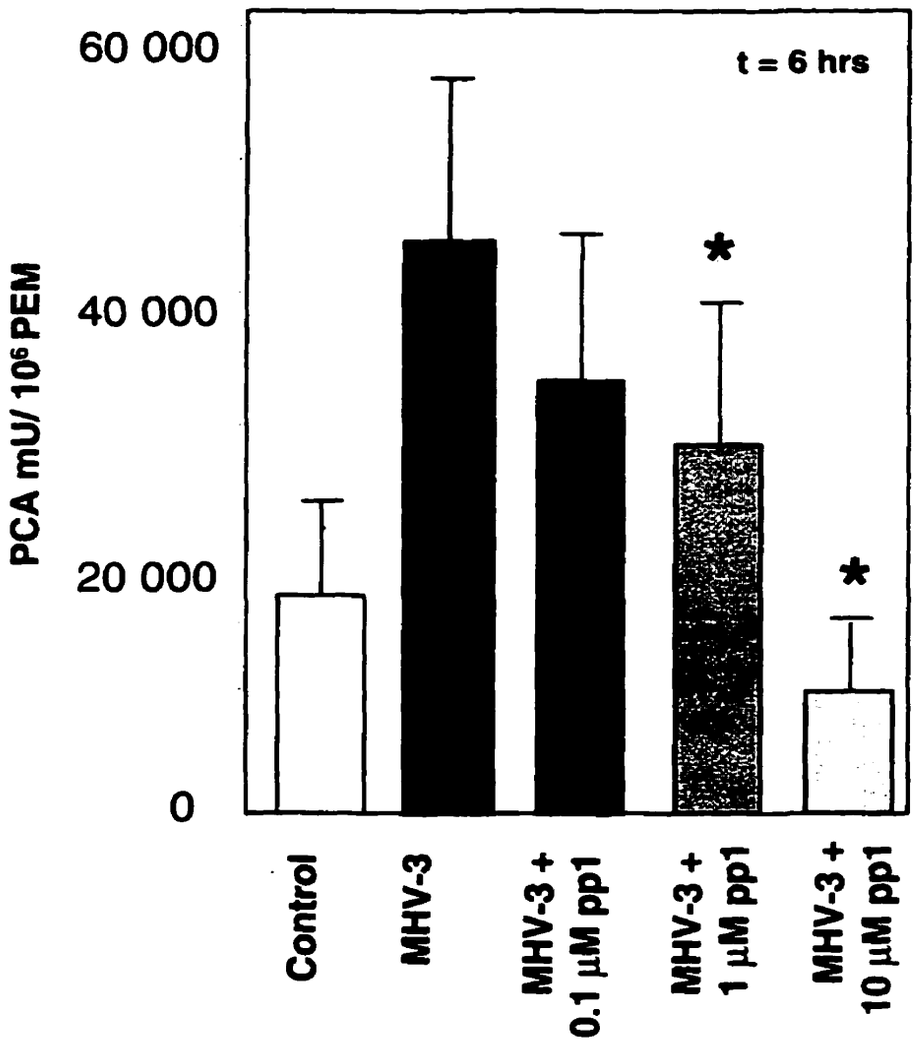

Figure 3.6. Src inhibition blocks MHV-3 induced procoagulant activity in a dose dependent manner. Cells (10^6/mL) were infected with MHV-3 (MOI 1) in the presence or absence of PP1 (0.1-10 μM) and incubated at 37°C/5%CO_2 for 6 hours. Cells were freeze thawed and assayed for procoagulant activity (PCA). In a dose dependent fashion, PP1 inhibits MHV-3 induced PCA (*p <0.05 compared to MHV-3 alone). The figure represents the cumulative results of at least 3 independent experiments, each done in duplicate.

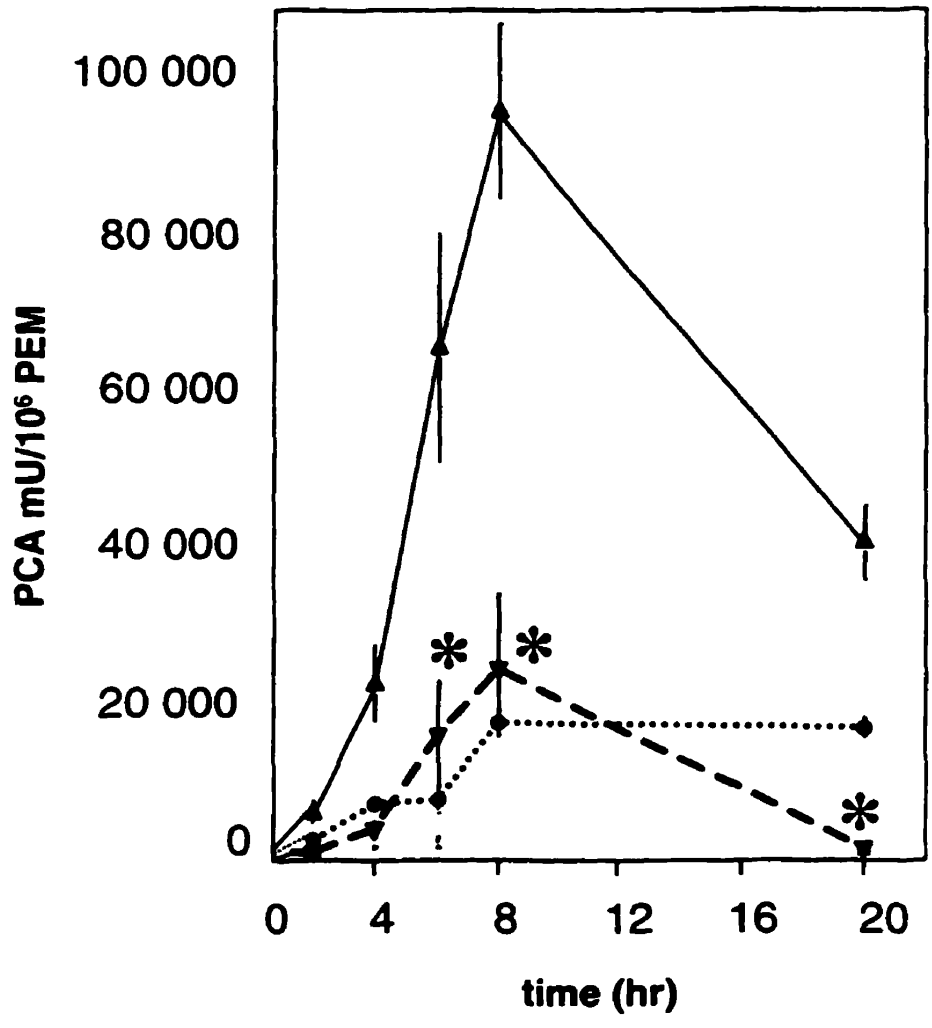

Figure 3.7. Src inhibition attenuates MHV-3 induced procoagulant activity in a time dependent manner. Cells (10^6/mL) were infected with MHV-3 (MOI 1) in the presence or absence of PP1 (10 μM) and incubated at 37°C/5%CO_2 for various times. Cells were freeze thawed and assayed for procoagulant activity (PCA). The solid line represents MHV-3 alone, the dashed line represents MHV-3 + 10 μM PP1, and the dotted line represents unstimulated PEM. PP1 treatment attenuates PCA with statistically significant inhibition at 6, 8 and 20 hours. (*$p < 0.05$ compared to MHV-3 alone). The figure represents the cumulative results of at least 3 independent experiments, each done in duplicate.

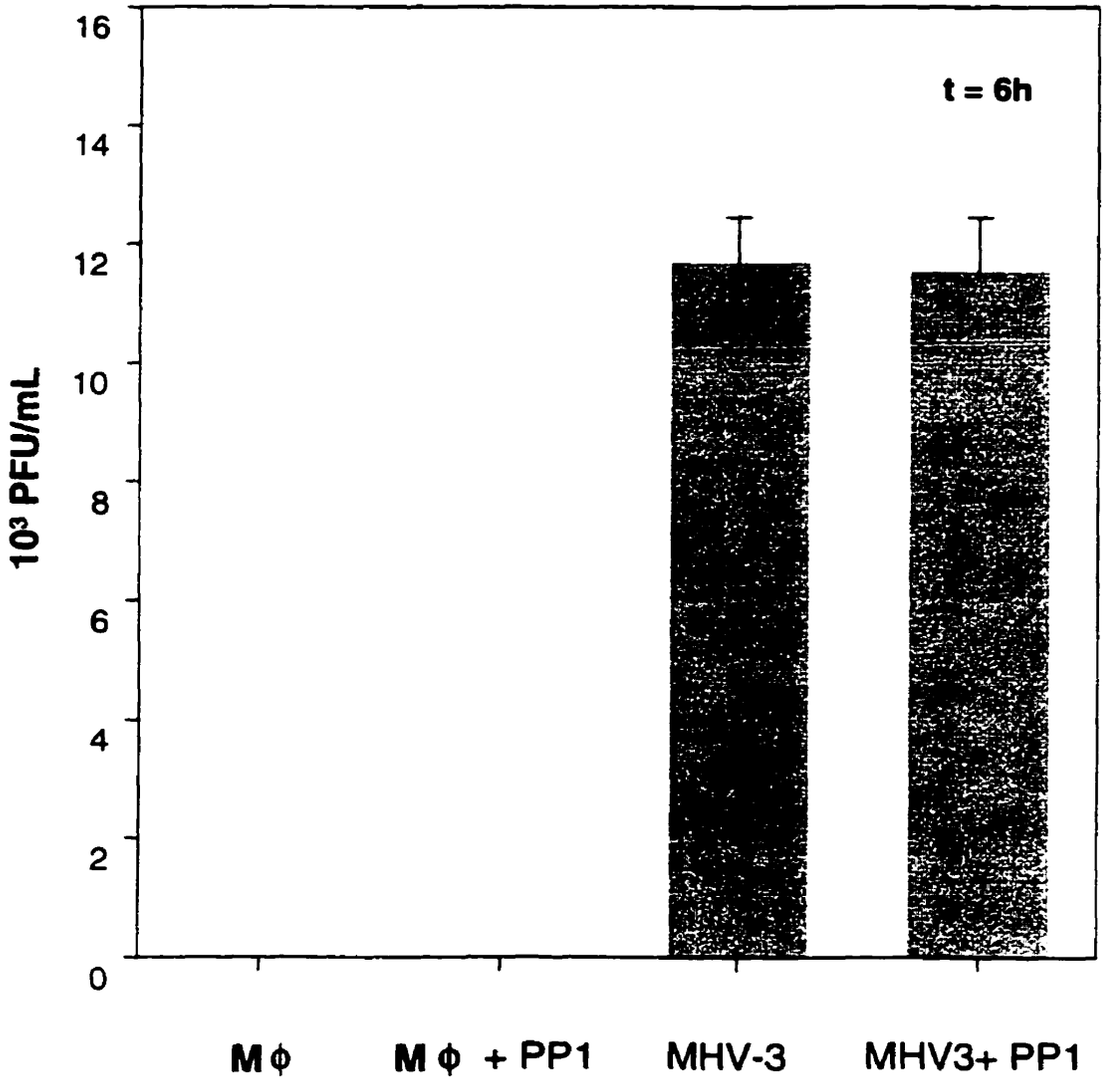

Figure 3.8. Src inhibition with PP1 does not affect MHV-3 replication. PEM (1.5 X 10^6) were infected with MHV-3 (MOI 1) and incubated at at 37°C/5%CO_2 for 10 hours. Some cells were treated with PP1 for 15 min prior to, and during the time of MHV-3 infection. Cells were freeze-thawed and MHV-3 levels were measured using a standard plaque assay on L2 cells. There is no significant difference in the ability of MHV-3 to replicate in PP1 treated or untreated cells.

To ensure that Src inhibition did not merely result in a delay of MHV-3 induced PCA, the effect of PP1 (10 μM) was examined over time (Figure 3.7). In MHV-3 infected PEM, maximal PCA was seen at 8 hours, declining gradually, though significant PCA remained at 20 hours after infection. Over the same time course, PP1 treatment of cells prevented MHV-3 induced PCA with PCA remaining near baseline levels. Statistically significant inhibition occurred at 6, 8 and 20 hours over MHV-3 infected cells ($p < 0.05$). Cell viability was unaffected at the doses of MHV-3 and PP1 used, as verified by trypan blue exclusion analysis. Interestingly, PP1 had no effect on viral replication over time (Figure 3.8). These data verify that the cells remained viable and that viral replication *per se* is insufficient for induction of PCA.

Src inhibition blocks p38 phosphorylation

We have previously shown that incubation of cells with MHV-3 caused both tyrosine phosphorylation and the activation of p38 and ERK1/2 (210), (153). Pharmacologic inhibition of p38 abrogates fgl-2 prothrombinase mRNA and protein expression, supporting a role for p38 in the signaling cascades leading to fgl-2 induction (154), (155). To discern the role of Src kinases in MHV-3 induced p38 activation, we tested the effects of Src inhibition on MHV-3 induced p38 activity.

While there was little constitutive p38 phosphorylation in control cells, MHV-3 treatment lead to a robust response, with p38 phosphorylation peaking at 20-30 minutes with reduction towards baseline at 60 minutes (Figure 3.9). When macrophages were pretreated with PP1, p38 phosphorylation was blunted in a dose and time dependent manner, with near complete inhibition at 10 μM PP1 (Figure 3.10). It is possible that the effects seen are a result of non-specific inhibition of the p38 pathway by PP1. However, other studies from our laboratory have shown that in other cell types, PP1 does not inhibit p38 activation. (239), (240). Studies by Rizoli *et al*

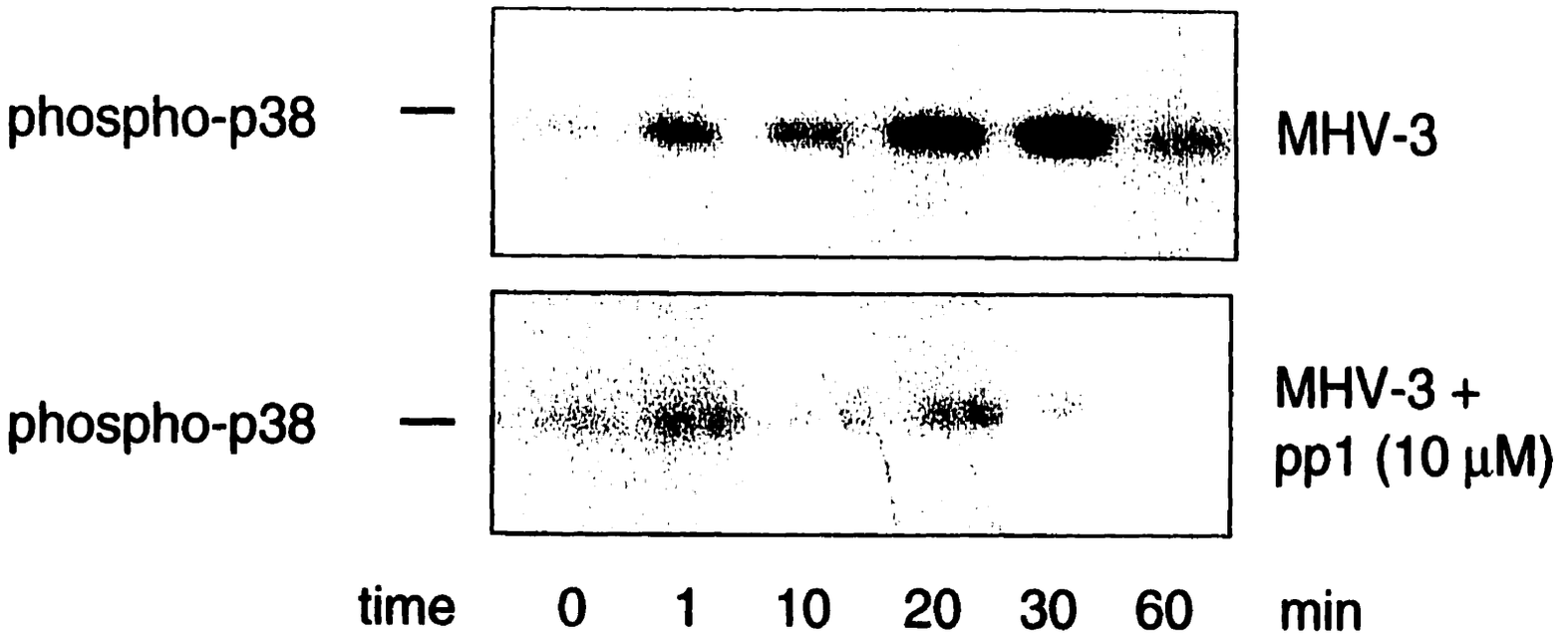

Figure 3.9. Phosphorylation of p38 MAPK by MHV-3 over time is inhibited by PP1. Cells (10^6) were pretreated for 15 min with PP1 (10 μM) then infected with MHV-3 (MOI 5) for various times at 37°C/5%CO_2. Whole cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against for phospho-p38 MAPK. The blots were then restripped and probed with an antibody directed against p38 to ensure comparable loading. Time course of MHV-3 induced phospho-tyrosine in the presence or absence of PP1 (10 μM). PP1 abrogates p38 phosphorylation at all time points. All lanes are from a single blot, which is representative of at least three independent experiments with similar results.

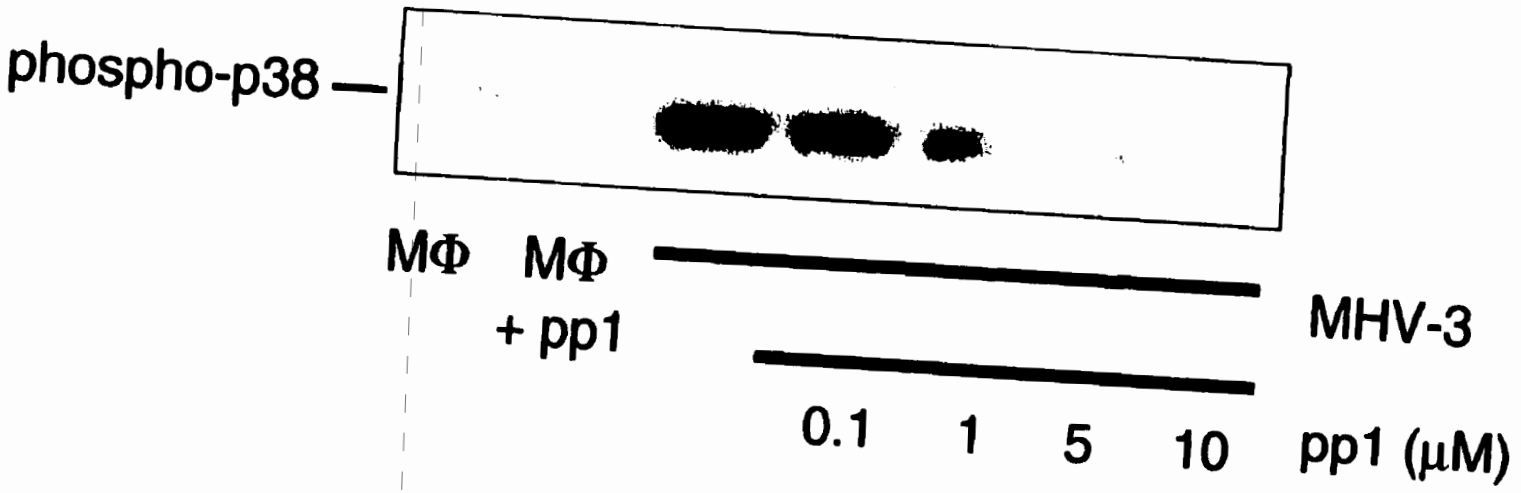

Figure 3.10. Phosphorylation of p38 MAPK by MHV-3 is inhibited by PP1 in a dose dependent manner. Cells (10^6) were pretreated for 15 min with various concentrations of PP1 (0.1-10 μM) then infected with MHV-3 (MOI 5) at 37°C/5%CO_2. Whole cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against for phospho-p38 MAPK. The blots were then restripped and probed with an antibody directed against p38 to ensure comparable loading. PP1 (0.1-10 μM) inhibits p38 MAPK phosphorylation in a dose dependent manner. The blots are representative of at least three independent experiments with similar results.

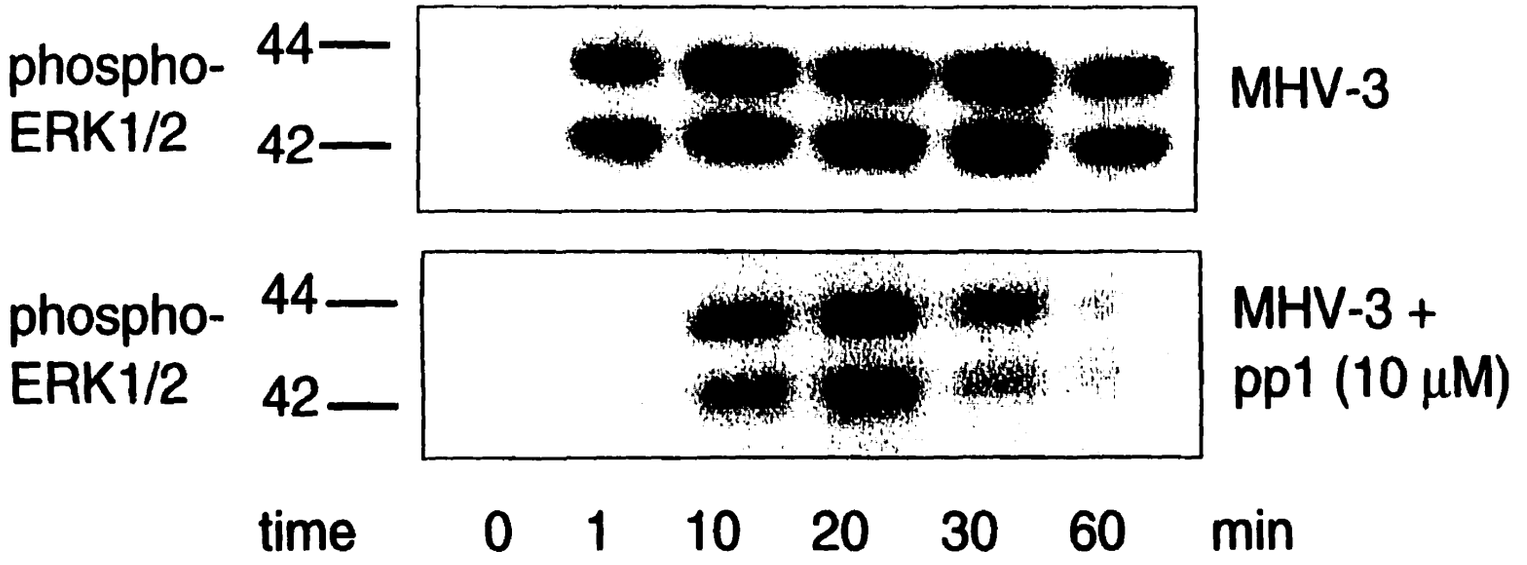

Figure 3.11. Phosphorylation of ERK1/2 by MHV-3 is attenuated by PP1 over time. Cells (10^6) were pretreated for 15 min with PP1 (10 μM) then infected with MHV-3 (MOI 5) for various times at 37°C/5%CO_2. Whole cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against phospho-ERK1/2. The blots were then restripped and probed with an antibody directed against ERK1/2 to ensure comparable loading. Time course of MHV-3 induced phospho-ERK1/2 in the presence or absence of PP1 (10 μM). PP1 partially inhibits ERK1/2 phosphorylation at all time points. All lanes are from a single blot, which is representative of at least three independent experiments with similar results.

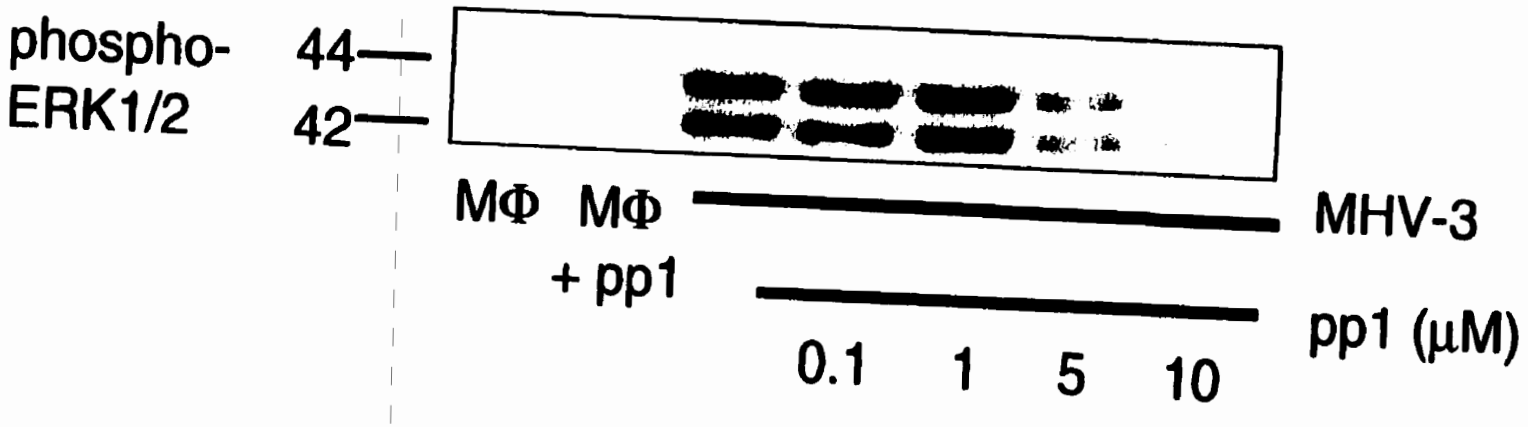

Figure 3.12. Phosphorylation of ERK1/2 by MHV-3 is inhibited by PP1 in a dose dependent manner. Cells (10^6) were pretreated for 15 min with various concentrations of PP1 (0.1-10 μM) then infected with MHV-3 (MOI 5) at 37°C/5%CO_2. Whole cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against phospho-ERK1/2. The blots were then restripped and probed with an antibody directed against ERK1/2 to ensure comparable loading. PP1 (0.1-10 μM) partially inhibits ERK1/2 phosphorylation in a dose dependent manner. The blots are representative of at least three independent experiments with similar results.

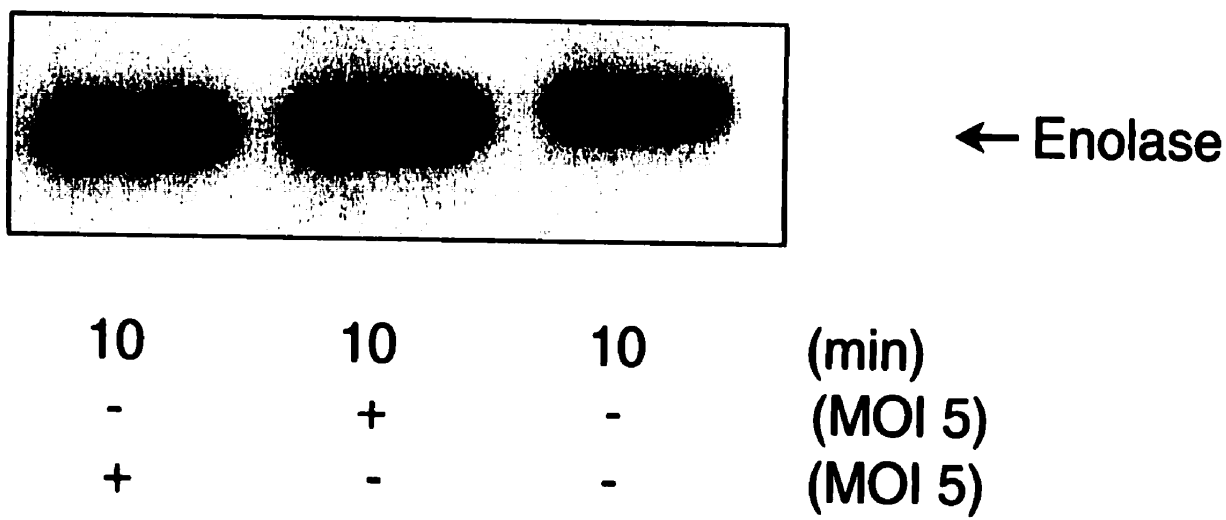

Figure 3.13. Different strains of MHV can activate Hck kinase. PEM (7.5 X 10^6) were infected with non fgl-2 inducing MHV-2 or fgl-2 inducing MHV-3 (MOI 5) for 10 minutes at 37°C/5%CO_2. Cells were pelleted and lysed with ice cold 1% Triton-X lysis buffer. Cytoplasmic Hck was immunoprecipitated and assayed for its ability to phosphorylate its substrate enolase. Proteins were then separated with SDS-PAGE and visualized by autoradiography. Hck activity is stimulated by MHV-3 at 10 min. MHV-2 infection leads to Hck activation at 10 min to similar levels as MHV-3. Uninfected cells from the same experiment are shown in the last lane.

have demonstrated in neutrophils, PP1 at the same doses used for these studies does not effect hypertonicity induced p38 phosphorylation or kinase activity (240). Thus, it is unlikely that the observed inhibition of p38 by PP1 in this system is due to non-specific p38 inhibition.

Src inhibition attenuates ERK1/2 phosphorylation

MHV-3 also induces the phosphorylation and activation of ERK1/2. In contrast to p38, fgl-2 mRNA expression was shown to be unaffected by pharmacologic inhibition of ERK. Although, functional PCA was blocked, suggesting a post-transcriptional role for this kinase (210). As shown in Figures 3.11-3.12, MHV-3 induces the phosphorylation of ERK1/2 by 1 minute after exposure, peaking at 30 min and remaining markedly elevated as long as 60 minutes. At concentrations shown to completely inhibit p38 phosphorylation, Src inhibition with PP1 caused a reduction in ERK1/2 phosphorylation

Activation of Hck occurs in different strains of MHV

Recent studies by Ning et al have examined the role of nucleocapsid protein in the induction of fgl-2 mRNA and protein. Deletions in the coding regions of the nucleocapsid protein of MHV were shown to account for the strain specific differences in fgl-2 induction. (211) We compared MHV-2, a non-fgl-2 inducing strain of MHV, to MHV-3 in their ability to activate Hck kinase. At 10 min of viral stimulation, non-fgl-2 inducing MHV-2 activated Hck kinase to a comparable extent as MHV-3 (see Figure 3.13).

IV. Discussion

The present studies demonstrate a role for the Src family kinases, Hck and Lyn in MHV-3 induced PCA and fgl-2 expression, and serves to further elucidate the signaling pathways emanating from viral binding to its receptor and the ultimate expression of the disease-evoking fgl-2 prothrombinase. MHV-3 infection of PEM induces early activation of Hck and Lyn kinase

activity without an effect on Fgr kinase. These kinases are involved in the induction of cell fgl-2 since pharmacologic inhibition of Src kinases markedly attenuates fgl-2 mRNA and functional PCA. Further, Src kinases lie upstream of ERK and p38 MAPKs in the fgl-2 signaling pathway, since PP1 treatment markedly attenuates the phosphorylation of both ERK and p38 MAPKs, which are required for functional fgl-2 PCA.

These findings provide some insights with respect to induction of fgl-2. The time frame of Hck and Lyn activation is on the order of minutes, much earlier that the time required for viral replication. This suggests that viral replication is not involved in the activation of Hck or Lyn. This adds to the evidence that viral replication is not involved in the initial signaling events required for fgl-2 expression. Indeed, clinical disease does not parallel viral replication, since viral replication occurs in both susceptible and resistant mice (50, 241). We have recently shown that tyrosine phosphorylation does not differ between replication deficient UV treated and untreated MHV-3 (210).

Interestingly, we found that two different strains of MHV, which differ in their nucleocapsid encoding region and in their ability to induce fgl-2, both induce Hck activity, suggesting that Src kinase activation is a necessary but not sufficient requirement of fgl-2 induction. Additional factors, including direct nucleocapsid protein interactions are required for full activation of fgl-2.

The involvement of Src in hematopoetic cell signaling is a well described motif. Upon stimulation, Lck associates with the cytoplasmic tail of CD4 in T cells (242), Fyn with the T cell antigen receptor (243), and Lyn with the B cell antigen receptor (243). In addition, Hck and Lyn are associated with the FcgRI (CD64) receptor on monocytes/macrophages (244).

More specifically, c-Src associates with human analogues to the MHV-3 receptor analogues such as human CD66 in granulocytes (162). Interestingly, CD66 has been shown to be

the receptor for *N. gonorrhoeae* bacteria and upon binding *of N. gonorrhoeae*, Hck and Fgr are activated (245). It is possible that MHV-3 binds to MHVR, resulting in Hck and Lyn association and activation, resulting in downstream target activation, culminating in fgl-2 PCA.

Further evidence for the role of Src family kinases in fgl-2 modulation comes from the fact that PP1 treatment dramatically reduces MHV-3 induced early tyrosine phosphorylation in multiple proteins. We have previously shown the importance of tyrosine phosphorylation for fgl-2 expression, since the tyrosine kinase inhibitor, genistein, prevents fgl-2 mRNA and PCA (153).

In this study, we delineated molecules with which Src kinases communicate. Src kinases have been implicated as upstream effectors of members of the MAPK family. Recently, conclusive evidence of Src regulation of the ERK pathway was described in which transfection of Csk, a negative regulator of Src kinases abolished oxidative stress activated ERK2 activity (246). Also, Src has been shown to be upstream of p38, since pharmacologic blockade of Src with PP1 prevented p38 activation by a constitutively activated G protein. In our system, PP1 attenuated, but did not completely abrogate ERK activation suggesting other inputs contribute to ERK activation. On the other hand, p38 MAPK was completely blocked by 10 uM PP1. Unlike our previous studies with selective p38 inhibition with SB20380, where p38 blockade prevented any induction of fgl-2, PP1 inhibition of the p38 pathway led to a 40% reduction in fgl-2 levels. Unlike SB20380, which directly inhibits p38 activity, PP1 prevents further activation of p38 kinase activity. It is likely that basal activity of p38, which is not inhibited by PP1, is capable of inducing a small amount fgl-2 mRNA.

However, it is clear that Src inhibition of ERK and p38 MAPK results in blockade of fgl-2 PCA. This may be due to post-translational changes in fgl-2 or possibly inhibition of a necessary co-factor for fgl-2 prothrombinase activity.

These results are important in defining the role of Src family kinases in the virally induced signaling by MHV-3, resulting in the induction of fgl-2 and acute viral hepatitis. Hck and Lyn kinases likely play a role in the physiologic signaling of the CD66 receptor, which is pathologically harnessed by MHV-3. It is also possible that the Src kinases interact directly with MHV-3 produced viral proteins such as the nucleocapsid protein.

Further, the ability of MHV-3 to induce Src mediated Pyk2 phosphorylation suggests a role Src mediated interaction with cytoskeletal structures. In osteoclasts, Src kinase activity is required for Pyk2 phosphorylation and formation of actin rich sealing zones, which are required for osteoclast function (237). Thus it is possible that Src plays a similar role in this system, modulating cytoskeletal organization through Pyk2. Further studies to explore these possibilities are warranted.

Src family kinases are a common target of viral modulation by pathologic activation or inhibition of Src kinase activity or expression of virally encoded Src analogues. Viruses such as Rous Sarcoma Viruses (RSV) encode v-src within its genome. The release of this viral protein causes cellular derangement and optimizes conditions for viral propagation. Some viral products interfere directly with Src function. For example, LMP2A protein of the Epstein-Barr virus associates with Src family of kinases (Fyn or Lyn) in infected cells and acts as a dominant negative regulator of Src by preventing B cell receptor signaling (247), (248). In contrast, mouse polyomavirus middle T-antigen associates with c-Src and activates it (249), (250). It is possible that other mechanisms exist for Hck and Lyn activation by MHV-3, such as through direct interaction between these Src molecules and MHV-3 encoded proteins. Further studies will be needed to clarify the upstream effectors of Src and the contributing effects of receptor engagement and direct interaction with viral proteins on Hck and Lyn activity.

As the signaling pathways of MHV-3 induced fgl-2 expression become clear, the goal is to understand the pathogenesis of acute viral hepatitis in this model and potentially of human fulminant viral liver failure. The human analogue of fgl-2 may play a role in the pathogenesis of this devastating disease, representing a final common pathway of immunologic activation. Targeted inhibition of the Src kinases, Hck and Lyn may represent a novel therapeutic modality for fulminant viral hepatitis.

CHAPTER 4: DISCUSSION

Activation of the coagulation cascade by inflammatory processes is involved in the pathophysiology of many disease processes. Tissue factor is activated in acute lung injury, (251), (252), bacterial abscesses, (253), (254), LPS induced liver injury (255) and autoimmune diseases such as lupus glomerulonephritis (256), (257). Infection with MHV-3 induces the expression of fgl-2, another procoagulant molecule, which represents another pathway for the activation of the coagulation cascade. Fgl-2 is induced by MHV-3 infection in macrophages and endothelial cells, and, leads to fibrin deposition, thrombus formation within hepatic sinusoids and ultimately results in hepatocellular necrosis. The paramount role of fgl-2 has been demonstrated in several studies, *in vivo* and *in vitro*, which have demonstrated that inhibition of fgl-2 by several methods attenuated or prevented procoagulant activity and/or liver damage. Recently, the human analogue of fgl-2, 'Fibroleukin', has been characterized and found to be secreted by CD3+/CD45R0+ memory T lymphocytes within the colonic lamina propria level (67). Other cells such as freshly isolated PBMC and virally transformed EBV-B cell lines also express fibroleukin (228), (67). The ability of virally transformed cells to induce fgl-2 in human tissue suggests a role for fgl-2 in virally mediated disease. This is further supported by the fact that IFN-γ, a cytokine involved in anti-viral immunity, can induce fgl-2 expression in murine peritoneal cells in the absence of viral stimulation (258).

In addition to its direct prothrombinase activity, it has been suggested that fgl-2, through its homology to fibrinogen may have adhesion properties, promoting cell-cell contacts or cell-ECM contact, possibly facilitating processes such as migration. As well, it may have cytokine functions. Fibrinogen modulates neutrophil activation by inhibiting neutrophil chemotaxis in response to zymosan and migration in response to fMLP (259). As well, fibrinogen is mitogenic for hematopoetic cell lines (260). It is possible that fgl-2 has cytokine effects since it is secreted by immunologically active T cells and macrophages at immunologically active sites such as the

lamina propria (261), (262), (263). Further characterization of fibroleukin will undoubtedly clarify the physiologic role of this molecule.

These studies reveal a key role for Src family tyrosine kinases in virally induced fgl-2 signaling. The ability of viruses to regulate Src activity is well established. In fact the first description of tyrosine phosphorylation in cells was in virally transformed Rous Sarcoma Virus infected cells (264), (265). The viral protein v-src was identified as a carboxy-terminus truncated form of the cellular protein c-src with proto-oncogene functions. Since the initial identification, viral interaction with Src proteins have been identified in several systems. In addition to RSV that encodes a truncated Src protein, proteins from several other viruses have been shown to modulate Src activity. Both activation and inhibition of Src activity has been observed (266), (267). The Nef protein of HIV-1 and the LMP2A protein of Epstein-Barr Virus inhibit Src kinase activity. The Nef protein of HIV-1 associates with Hck and Lck. Nef interacts with Lck is through its SH3 domain and leads to downregulation of Lck dependent tyrosine phosphorylation and Il-2 promoter activity in Jurkat cells (268). Similarily, LMP2A protein inhibits Lyn in B cell signaling through SH2 association leading to downregulation of sIg signaling and viral persistence (266). Other viral protein such as mouse middle T polyomavirus proteins bind and activate c-Src, c-yes and c-fyn though association with the SH1 domain leading to cellular transformation. Thus, Src kinases are a common target for viral interaction and viruses use different strategies to modulate Src kinase activity.

Strategies involving direct interaction with the regulating domains of Src have been described. As well, indirect effects on Src activity are likely present.(269) The mechanism by which MHV-3 regulates Hck and Lyn is unknown. It is unlikely that MHV-3 regulates Hck and Lyn through direct interaction between a viral protein and Hck or Lyn because the time frame of activation is short, on the order of minutes, thus likely does not require viral replication or

transcription of new proteins. Rather, it is likely that Hck and Lyn activation by MHV-3 is a result of early upstream signaling events, possibly due to receptor engagement by MHV-3.

The cellular receptor for MHV-3, MHVR (also known as C-CAM, CD66a), belongs to the CEA family of molecules, a complex family of molecules with several physiologic - properties. They participate in intercellular homophillic and heterophillic adhesion processes. Human C-CAM and the related nonspecific cross-reactive antigen (NCA), are ligands for E-selectin and are involved in the activation of endothelial-neutrophil β2- integrin mediated adhesion (270).

As well, C-CAM molecules are known to be pathogen receptors, not only for MHV-3 but also for bacterial pathogens such as *Neisseria gonorrhoeae* (245). Hauck *et al* demonstrated that *Neisseria gonorrhoeae* used C-CAM to enter human neutrophils and CD66 engagement was associated with downstream signaling events. Specifically, Hck and Fgr tyrosine kinases were activated. Further, the JNK MAPK, was also activated by C-CAM engagement. In a similar fashion, the engagement of MHV-3 to its receptor may be required for viral entry and may be the crucial initiator of signaling events, which eventually result in the activation of intermediary signaling proteins, Hck, Lyn tyrosine kinases and the MAPK kinases p38 and ERK, that are required for fgl-2 induction and activity. In addition to the studies involving *Neisseria gonorrhoeae* activation of C-CAM several other groups have described an association between C-CAM and cytoplasmic tyrosine kinases. In human neutrophils activated by fMLP, PAF or PMA, Lyn and Hck tyrosine kinases were co-immunoprecipiated with C-CAM and may play a role in the function of C-CAM (163). In another study, C-CAM was shown to be co-immunoprecipitated with Src kinase in human neutrophils and in the colonic cancer line HT29. In this system, the association was found to be between the SH2 domain of Src and the tyrosine phosphorylated cytoplasmic domain of C-CAM. In the MHV-3 system, in addition to the

findings of these series of experiments, the ability of replication deficient UV irradiated MHV-3 to trigger tyrosine phosphorylation further supports the hypothesis that receptor engagement may be sufficient to initiate early signaling events. Further, the ability of non-fgl-2 producing MHV-2 to activate Hck kinase support the notion that early signaling events, possibly in response to receptor engagement, can result in signaling events that are necessary but not sufficient for fgl-2 induction.

These studies have demonstrated a role for the Src family of tyrosine kinases in MHV-3 induced activation of the hepatitis associated procoagulant molecule fgl-2. Hck and Lyn kinases are shown to be activated in response to MHV-3 stimulation and this activation is required for fgl-2 induction, since specific inhibition of Src kinase activity attenuated the expression of fgl-2 mRNA and protein. Further, Src inhibition markedly attenuated the activity of p38 and ERK, demonstrating that Hck and Lyn lies upstream of these kinases in the early signaling pathway of MHV-3 induced tyrosine phosphorylation. Thus, MHV-3 stimulates Hck and Lyn kinases, which lead to p38 and ERK activation, leading to fgl-2 induction and activity. In addition to elucidating the signaling pathways used by MHV-3, these studies also contribute to the understanding of viral pathogenesis, specifically in relation to viral hepatitis, and more generally, by demonstrating viral modulation of Src activity. These experiments demonstrate a motif used by several viruses, the modulation of Src activity. In this system, MHV-3 activation of Src kinases is involved in the pathogenesis of clinical disease. As well, it supports the emerging view of Src kinases as upstream regulators of MAPK pathways. Many lines of evidence in several systems have implicated Src in p38 and ERK activation.

More specifically to viral hepatitis, these studies contribute to the understanding of the mechanism of fgl-2 induction and the pathogenesis of fulminant liver failure. The activation of the procoagulant molecule fgl-2 may represent a final common pathway of immunologic

derangement, thus a full understanding of the activation pathways of fgl-2 may provide a foundation for specific therapeutic interventions.

I. Directions for future research

These experiments have demonstrated a role for Src kinases in the signaling pathways induced by MHV-3. However, these studies are an early step in the full understanding of the signaling process that eventually results in the expression of fgl-2. These experiments have generated several unanswered questions that should be addressed in future studies.

The upstream and downstream components of the pathway leading to Hck and Lyn activation are as yet unidentified. As well, the mechanism by which Src activates p38 and ERK is not known. Future experiments are required to determine mechanism of Hck and Lyn activation. The role of MHVR receptor engagement in the signaling pathways should be examined since several studies have shown association between related C-CAM receptors and the Src kinases. These could be examined by co-immunoprecipitation studies using antibody based systems. As well, other upstream regulators of Src should be examined. For example the role of CSK, a negative Src family regulator within this system could be examined. Other experiments to determine the specific mechanism of Src activation could be performed. The regulation of Src activity is multipronged and includes changes in subcellular localization, conformational changes due to binding of the SH2 and SH3 domains as well as changes due to phosphorylation. The mechanisms used by MHV-3 to activate Hck and Lyn are unknown. As well, the specific roles of Hck and Lyn kinases within this system are unidentified. Experiments using single Hck or Lyn gene knock-out systems, examining endpoints such as PCA and clinical parameters of hepatitis, would provide information about the necessity of each kinase.

As well, the immediate downstream targets of Src and the signaling pathways between Src kinase activity and p38 and ERK kinases are not delineated. Thus, experiments in this regard

would improve the understanding of the induction of fgl-2. In these experiments, MHV-3 was shown to phosphorylate the Src substrate Pyk2. Thus, the effects of MHV-3 on Pyk2 function may contribute further to the understanding of MHV-3 pathogenesis.

Several questions regarding fgl-2 also remain unanswered. The virally independent activation of fgl-2 is poorly understood. It is known that fgl-2 is expressed in macrophages by high dose interferon treatment in the absence of MHV-3. Thus, experiments looking at the role of Src kinases, p38 and ERK in interferon dependent signaling pathways leading to fgl-2 production could be performed. This would identify whether the effects on Hck and Lyn can be generalized to other fgl-2 initiating conditions.

As well, in vivo experiments using Src kinase inhibition could be performed to see whether inhibiting this important pathway could prevent MHV-3 induced liver disease. These experiments would demonstrate the absolute requirement for Src in MHV-3 induced disease and may stimulate development of novel treatments for fulminant viral hepatitis.

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