STUDIES ON HOST FACTORS THAT REGULATE THE REPLICATION OF POSITIVE

STRAND RNA VIRUSES

by

JOHN B PATTON

B.A., Tabor College, 2003

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

Abstract

Positive sense RNA viruses include a diverse group of pathogens that cause a wide array of diseases that can range from sub-clinical to lethal. These viruses infect humans and mammals as well as a variety of other hosts. For their successful replication, viruses interact closely with host cells from the binding to the receptor to the exit as complete viral progenies. During the events, viruses are dependent on host factors for receptor bindings, genome synthesis, and trafficking of viral genome and proteins. Thus there have been major efforts on the studies of understanding the virus-host interactions in the field of virology. In my PhD program, I have studied the host factors that regulate the replication of viruses using porcine reproductive and respiratory syndrome virus (PRRSV) and hepatitis C virus (HCV). I found that modulation of either the viral receptor or cellular signalling pathways had pronounced effects in the replication of PRRSV or HCV respectively. Using PRRSV, I found that the modulation of the level of the putative receptor CD163 on cells with cytokines significantly influence virus replication, suggesting the importance of cytokine presence in environments to determine the replication and pathogenicity of PRRSV via receptor expression *in vivo*. With HCV, I found that the enhancement of the virus replication occurs through the activation of the epidermal growth factor receptor/extracellular signal-regulated kinase pathway by bile acids which are abundant in the liver where the virus targets *in vivo*. Furthermore, I found that the bile acid-mediated signalling pathway significantly inhibited the antiviral activities against HCV. These results indicate the importance of environmental factors such as bile acids and signalling pathways in the replication and pathogenicity of HCV *in vivo*.

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Positive sense RNA viruses include a diverse group of pathogens that cause a wide array of diseases that can range from sub-clinical to lethal. These viruses infect humans and mammals as well as a variety of other hosts. For their successful replication, viruses interact closely with host cells from the binding to the receptor to the exit as complete viral progenies. During the events, viruses are dependent on host factors for receptor bindings, genome synthesis, and trafficking of viral genome and proteins. Thus there have been major efforts on the studies of understanding the virus-host interactions in the field of virology. In my PhD program, I have studied the host factors that regulate the replication of viruses using porcine reproductive and respiratory syndrome virus (PRRSV) and hepatitis C virus (HCV). I found that modulation of either the viral receptor or cellular signalling pathways had pronounced effects in the replication of PRRSV or HCV respectively. Using PRRSV, I found that the modulation of the level of the putative receptor CD163 on cells with cytokines significantly influence virus replication, suggesting the importance of cytokine presence in environments to determine the replication and pathogenicity of PRRSV via receptor expression *in vivo*. With HCV, I found that the enhancement of the virus replication occurs through the activation of the epidermal growth factor receptor/extracellular signal-regulated kinase pathway by bile acids which are abundant in the liver where the virus targets *in vivo*. Furthermore, I found that the bile acid-mediated signalling pathway significantly inhibited the antiviral activities against HCV. These results indicate the importance of environmental factors such as bile acids and signalling pathways in the replication and pathogenicity of HCV *in vivo*.

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Dedication

I would like to dedicate this dissertation to my wife Dr. Karen Patton. Without her patience and constant support I could not have completed this research.

CHAPTER 1 - Literature Review and Significance

RNA viruses are a wide ranging and diverse group of viruses that inhabit many different hosts and cause diseases ranging from the non-clinical to deadly (8, 215). Within the past several decades a number of RNA viruses have emerged around the world infecting both human and domestic animal populations (86, 102, 158, 199). Both enveloped and non-enveloped RNA viruses have been identified. RNA viruses are classified based upon the composition of their RNA genome. The genomic structure of the RNA virus can be either positive or negative sense single stranded ribonucleic acid (RNA) or double stranded RNA. The RNA genome from a positive sense virus is considered to be infectious and can be directly translated by the host proteins. There currently exist a number of RNA viruses that no effective vaccine or antiviral treatment has been identified. Within the last several decades several new RNA viruses have emerged onto the world scene. These new viruses are continuing to cause both extensive economic losses as well as the loss of life.

In the early 1990's a new virus began rapidly spreading through the swine herds of both Europe and the United States. This virus originally identified by names such as blue ear disease and mystery swine disease but has more recently been termed the Porcine Reproductive and Respiratory Syndrome (PRRS). The causative agent was identified as a positive sense RNA virus that was later determined to be an arterivirus (86, 199). In 1963 scientists developed a test to identify Hepatitis B shortly followed by Hepatitis A tests in 1973 (54, 196). Unfortunately, scientists still had not identified the causatative agent of a new post-transfusion illness that they had termed Non-A/Non-B hepatitis. It was not until the 1980's researchers finally isolated the

causative agent of Non-A and Non-B hepatitis (34). Hepatitis C Virus (HCV) is now known to be a worldwide health concern with around 3% of the worlds population persistently infected (2).

The recent outbreaks of multiple unique viruses highlight the need for a better understanding of viral host cell interactions. A better understanding of these interactions may help to significantly improve the development of antiviral treatments and vaccines. Within the research for this dissertation I analyzed the effects of the regulation of several host factor on the replication levels of both porcine reproductive and respiratory syndrome virus (PRRSV) and HCV. We demonstrated that modulation of either the viral receptor CD163 or the ERK pathway had pronounced effects in the replication of PRRSV or HCV respectively.

PRRSV Virus

Porcine Reproductive and Respiratory syndrome (PRRS) was originally identified in the Netherlands in 1987 and later in the United States in 1990 (86, 199), and has sense been identified as an economically important disease in swine costing farmers over 650 million dollars a year in the United States alone (136). Before the identification of the PRRS virus and the finalization of the disease name, PRRS, the disease had been identified by several different names within both North American and Europe including blue eared pig disease, mystery swine disease, and mystery reproductive syndrome.

Currently the PRRS virus is broken into two distinct genotypes. The first genotype consists of the North American strains (Type II), characterized by the reference strain VR2332, and the second genotype is comprised of the European strains (Type I), characterized by the Lelystad

virus. Recently a number of Type I like viruses have been identified within the United States and have been termed Euro-like strains. Phylogenetic analysis has determined that there is only a 60% nucleotide homology shared between these two clades of viruses and antibodies between types I and II are not cross protective (62, 69, 135, 149).

The current PRRSV vaccines only have a limited efficacy and have been demonstrated to revert to virulence. The current modified live vaccines are not cross protective between the North American strains and European strains (140). In the 1990's Denmark initiated a vaccination program, vaccinating a large population of the swine industry with an attenuated North American virus (21). Unfortunately, a portion of the animals that were vaccinated had the attenuated virus revert to virulence (21). Originally, PRRSV could be isolated from infected animals using pulmonary alveolar macrophages (PAMs) (71). Studies were hampered by the lack of an immortal cell line supporting PRRSV replication. In 1983, a sub clone of MA-104 cells was identified in which both the European and North American strains replicated to high titers, this sub-clone was identified as MARC-145 (13, 89).

Several putative receptors for PRRSV have been identified on MARC-145 and PAMs including sialic acid, sialoadhesion, vimentin, and CD163 (29, 45, 46, 89, 190). In recent studies Calvert *et al.* demonstrated that cells that support viral replication but that are not permissive to PRRSV infection, can be rendered permissive by the stable transfection of CD163. Further they demonstrated that the stable transfection of CD163 was able to generate permissive cell lines even in the absence of sialoadhesion (29). While screening the cDNA library for the PRRSV receptor Calvert *et al.* used a recombinant PRRSV virus that encoded the gene for the green

fluorescent protein (GFP) within its genome allowing for the easy identification of infected cells. The incorporation of this foreign protein was accomplished using a reverse genetic system, or infectious clone.

Genome manipulation of an RNA virus is exceedingly difficult without the use of an infectious clone. Infectious clone have been developed for each of the representative strains of PRRSV, VR2332 and Lelystad (91, 117, 214). These infectious clones allow for the exploration of individual viral proteins, the incorporation of separate subgenomic open reading frames for foreign proteins, and the deletion of portions of the viral genome for the creation of DIVA assays. The incorporation of foreign tags, similar to the GFP recombinant virus outlined above allow for the creation of marked vaccines. These vaccines allow for the identification of vaccinated animals, although they do not permit the differentiation between vaccinated and naturally infected animals (29).

Hepatitis C Virus

Hepatitis C Virus (HCV) was originally known as hepatitis of non A or B origins (34).   HCV is currently a worldwide health problem with more than 170 million chronically infected individuals worldwide, approximately 3% of the worlds total population (2). The World Health Organization (WHO) estimates that there are an additional 3 to 4 million patients infected annually of which only 25% show symptomatic infections (3). Unfortunately, of those 25%, 60 to 80% will develop chronic infections. In up to 20% of these chronically infected patients the HCV infection will result in liver cirrhosis (114). HCV is currently the leading cause of liver transplants in the United States accounting for over 2/3rds of the liver transplants (126). The

causative agent was later identified as a positive sense, enveloped RNA virus belonging to the Flaviviridae family. Through phylogenetic analysis 6 different genotypes of HCV around the world have been identified each consisting of a number of subtypes. The predominant genotypes found within the US are genotype 1 and 2 (3).

Each of the 6 genotypes show different response rates to the current standard treatment for HCV infection which consists of a combination therapy of high dose pegylated IFN-α and Ribavirin (27, 202, 213). This therapy has been shown to be highly effective in the genotype 2 and 3 viruses with upwards of 80% of patients showing a sustained virological response after the termination of treatment. Unfortunately, genotype 1 viruses do not respond as well to the current treatment regime with only 40 to 50 percent of patients demonstrating a sustained virological response after treatment. There have been documented cases where a number of patients infected with genotype 1 viruses were complete non-responders to treatment (105).

The continued development of treatments for HCV has been hampered with the lack of any genotype 1 or 2 tissue culture adapted viruses. Within the last several years with a representative strain of both genotype 1 and 2 viruses being tissue culture adapted and the isolation of these two representative strains is a significant breakthrough in the field of HCV research (101, 192, 211, 212, 218). Unfortunately, the vast majority of HCV virus strains remain fastidious in tissue culture. Prior to the availability of tissue culture adapted HCV viruses researchers often relied in replicon-harboring cells. Stable cell lines were developed expressing either the non-structural proteins or entire genome of HCV. These cell lines containing an HCV replicon proved invaluable to researchers attempting to develop antivirals or further understand the replication

cycle of HCV. One of the major remaining benefits of the HCV replicon-harboring cells over the tissue culture adapted viruses, is the fact that they are available in a wide variety of genotypes and sub-genotypes allowing the comparison of treatments across HCV strains (10, 102, 184).

Using these replicon-harboring cells researchers have focused on two main directions in the development of antivirals against HCV infection. The first involved the targeted development of antivirals towards the individual viral proteins, and the second, targeting the host cell pathways and proteins that are used during viral replication.   The development of many of the antivirals against HCV has been directed at the NS3-4a and NS5b viral proteins (52, 139). These proteins are responsible for the viral protease and RNA-dependent RNA-polymerase of HCV. A number of candidate antivirals have been identified with several in clinical trials. Unfortunately, the rapid rates of mutation in RNA viruses often lead to the generation of non-susceptible mutants that these drugs are no longer effective against (163). The second group of antivirals being developed specifically targets host cell factors that are necessary for viral replication. Targeting these cellular factors partially removes the problem associated with viral targeted antivirals although resistant mutants have been isolated. The main drawback to this approach is the targeting of host cellular pathways can cause higher toxicity side effects (64, 79, 104, 152).

Viral Entry and Uncoating

Many positive sense RNA viruses including both PRRSV and HCV bind to their viral receptor being displayed on the cell surface. They are then trafficked into a clathrin coated pit for endocytosis into the host cell. After endocytosis they are trafficked to the early endosome where they begin their uncoating process. Depending upon the virus they escape the endosomes at

different points either early endosome or late endosome. PRRSV escapes from the early

endosome, and begins to replicate within the cytoplasm of the host cell (124, 188). HCV also

escapes from the early endosome by fusion of its viral membrane to the membrane of the

endosome releasing the viral core into the cytoplasm (18, 60, 106).

Viral Receptors

Viral receptors can be as varied and diverse as the viruses themselves. Currently the overall

knowledge of the viral genome replication, transcription and packaging far outweighs our

knowledge of viral receptors, although this field of study is rapidly growing.   Multiple methods

have been used to identify viral receptors including electron microscopy (EM), enzymatic assays,

competitive based binding assays, cDNA library screening, and monoclonal antibody libraries.

The first methods of identifying viral receptors were both slow and tedious. They relied heavily

on the use of EM and enzymatic methods for the characterization of each individual receptor.

Recent advances in both genetic and immunologic fields have improved our ability to identify

viral receptors. The development of monoclonal antibodies libraries against entire cells,

transfection techniques, and the ability to express soluble forms of receptors from cloned cellular

genes has allowed some significant advances in the identification of viral receptors. Although

these advances have improved our ability to identify these receptors there are still a number of

difficulties in deducing the primary receptor and co-receptors (61).

Currently a number of viruses have had their cellular receptors and coreceptors identified

through the use of multiple techniques. The Polio receptor was identified after transfection of

resistant mouse cells with a cDNA library derived from human cells. A gene called Pvr was

identified as a molecule which could make the mouse cells susceptible to Polio infection. For a brief period it was also believed that CD44 played a role as a co-receptor for polio because anti-CD44 antibodies could block Polio infection. It was later determined that CD44 is found in the same lipid rafts as Pvr and the monoclonal CD44 antibodies that had bound CD44 were interfering with the binding of Polio to Pvr within the same lipid rafts (153).

ICAM-1 the rhinovirus receptor was identified through the high throughput screening of thousands of antibodies looking for an antibody that could block viral infection. Blocking of the ICAM-1 surface receptor was found to block the infection of cells by the majority of different serotypes of rhinovirus. ICAM-1 is a member of the IgG gene superfamily consisting of 5 IgG folds (15). This molecule has since been shown to be the single molecule responsible for virus binding and entry (39).

Methods of Identification

A number of different methods have been employed to identify viral receptors. Beginning in the 1960's the binding of purified virions to host cells was observed using electron microscopy. Using these images researchers attempted to deduce what receptors played a role in viral entry. EM techniques have successfully determined the viral binding sites i.e. polarized cells or specific tissue types, but unfortunately, using EM to help deduce the receptors for a virus is often problematic due to the low resolution at that scale. Using EM for receptor identification is complicated due for multiple reasons including the low resolution and the inability to differentiate between viruses binding to a specific viral receptor and a non-specific interaction (61).

When determining the viral receptor using enzymatic methods, host cells are treated with varying enzymes that are known to cleave specific molecules or families of molecules from the cell surface. After enzymatic cleavage of the surface molecules the cells were subjected infectious virus and the resulting infection was monitored. Populations of cells that are resistant to infection suggest putative viral receptors. Further enzymatic studies are then used to isolate individual putative viral receptors.

The process of receptor identification has been further refined in recent years through the use of cDNA libraries, monoclonal antibodies, complementation assays, and other techniques. The identification of viral receptors from cDNA libraries relies on a complete cDNA library generated from the permissive cell lines (29). The resulting plasmid library is then either size fractionated or otherwise sorted before transfection into a cell line that supports replication but not viral entry. Once a group of plasmids that confers susceptibility is isolated it is further fractionated until the appropriate clone is isolated. This clone can then be further analyzed to determine what gene it encodes. The use of cDNA libraries has allowed for the identification of multiple viral receptors including PRRSV and polio. Polyclonal antibody libraries against whole cells have been generated as a means of identifying cellular receptors required for viral infection. Similar to the cDNA libraries these polyclonal libraries are fractionated and their ability to block viral infection is quantified (127, 198).

Modulation and Disruption of Viral Receptors to Alter Virus Replication

The modulation or disruption of the viral receptors can reduce or abolishes the ability of that virus to enter the host cells to begin replication. Treatment of cells with small molecule

inhibitors, siRNA's, transfection of dominant negative mutants are all effective ways of reducing the overall expressing and/or function of the viral receptor. Modulating or disrupting the cellular receptor for the HCV virus CD81 using siRNA's have been shown to block HCV infection using an HIV-HCV pseudo-type virus (217).

PRRSV Virus Receptor

There are currently a number of putative PRRSV receptors that are detailed in the literature. Beginning in 1998, Duan *et al* (48), began exploring the cellular surface antigens that induce cell susceptibility to the PRRSV virus. They developed a series of anti-PAM and anti-PBMC polyclonal antibodies and determined that the anti-PAM antibodies and not the anti-PBMC antibodies could protect PAMs from PRRSV infection. Two antibodies 41D3 and 41D5 were show to bind specifically to PAMs and not other porcine cells, and had the ability to neutralize PRRSV infection (48).

This was followed in 2000 when Therrin *et al* (183) began to characterize the binding factors of PRRSV. It was shown that PRRSV can bind both PAMs, and MARC-145 cells. It was also noted that PRRSV has the ability to bind to porcine peripheral blood monocytes but showed no evidence of replication. Further studies confirmed that the only peripheral blood monocytes and macrophages but not leukocytes could bind the virus. They were able to block the infection of PRRSV by preincubation of the cells with either chymotrypsin or pronase E, after 8 hours in culture the cells regenerated the ability to support PRRSV infection. The results suggest that PRRSV can interacts with a specific receptor on the surface of some cells but this interaction is not enough to lead to a productive infection (183).

In 2002 Delputte *et al* (46) extended this work by further narrowing down the list of possible receptors when they identified that heparin could interact with the PRRSV virus and reduce infection of PAMs by 88 or 92% overall, depending on the genotype of PRRSV (American or European respectively). When virus was treated with glycosaminoglycans, which are similar to heparin, there was no significant impact on the overall levels of viral infection. Treatment of cells with heprinase significantly reduced the overall levels of cells infected with the PRRSV virus. The M-GP5 heterodimer appears to play a role in the attachment of a heparin-like receptor on PAMs. The research conducted by Delputte was the first to narrow down the possible PRRSV receptors to a specific group of proteins (46).

Wissink *et al* (201), identified multiple glycoproteins that they believed were responsible for PRRSV infection in PAMs. Their work centered on a group of four monoclonal antibodies that reacted with protein on the surface of PAMs and were known to block PRRSV infection. These antibodies: 1-8D2, 9.4C7, 9.9F2, and 3-3H2 were shown to be specific to PAM surface antigens through the use of immunoflorescence and immunoperoxidase monolayer assays. Using radioimmunoprecipitation assays they identified two separate proteins one 150kDa protein and a second 220kDa protein that reacted with the monoclonal antibodies. Glycosidase treatment proved that both of these proteins were N-glycoslyated (201).

Further extending the work of Delpuette *et al*. (49), Vanderheijden and colleges (190) immunoprecipitated a 210kDa protein (p210) and after tryptic digestions demonstrated a 56 to 91% homology with mouse sialoadhesin and human sialoadhesion respectively. When the cDNA for p210 was cloned into PK-15 cells, the PK-15 cells internalized both American and

European strains of the virus through glycoprotein mediated uptake. They suggested that the ability of porcine sialoadhesion to mediated endocytosis of PRRSV virus implicates that it is involved in the entry process of PRRSV.

Screening of a cDNA library derived from the total RNA of PAMs Calvert *et al.* (29) identified CD163, a 130kDa protein, that was capable of generating permissive cell lines from previously non-permissive ones. The research showed that CD163 from human, monkey, porcine, mouse, or canine cells was capable of encoding a functional viral receptor. They generated a number of stable cell lines that were susceptible to PRRSV infection through 80 serial passages (29). CD163 may correspond to the 150kDA protein that was originally identified by Duan in 1998 (48).

Follow up papers by Van Gorp H *et al* (187) suggest that CD163 and sialoadhesin play a cooperative role in the binding and internalization of the PRRSV virus. They noted that monoclonal antibodies to either sialoadhesin or CD163 could reduce PRRSV infection in PAMs by up to 75%. We have also shown the reduction of PRRSV infection levels using CD163 monoclonal antibodies. Confocal analysis showed that PRRSV was internalized in the presence of sialoadhesion alone, but never successfully uncoated. Full PRRSV endocytosis and uncoating was only observed in the presence of both sialoadhesion and CD163 (187-189).

Hepatitis C Virus Receptor

There are currently a number of putative receptors for HCV that have been identified. The putative receptor that has become the most accepted and most researched is CD81. CD81, a

25kDa protein, is a member of the tetraspanin family and was the first putative receptor identified for HCV. As the family name suggests CD81 consists of 4 membrane spanning domains, these are connected through 2 extra cellular loops. HCV E2 protein appears to interact with the large extra cellular loop which is conserved between the only 2 known hosts of HCV, humans and chimpanzees. The interaction site of E2 and CD81 has been mapped to residues 163, 186, 188, and 196 of CD81. Currently the E2 domains that bind to CD81 are being debated. Four different domains are hypothesized as being the binding domains of E2. The first pair involve aa 480-493 and 544-551 and the second involve aa 612-618 and 384-410 in concert with 476-480. Similar to the PRRSV virus receptors detailed above there is some evidence that multiple cellular receptors are required for the entry of HCV (11, 70, 72).

In 1999 two groups proposed a second mechanism for the entry of HCV into the host cell. Agnello *et al.* (4) and Monazahian *et al.* (125) reported observations that the HCV virus bound low density lipoprotein (LDL). After binding to LDL it was shown that HCV entered the cell through endocytosis of the LDL mediated by the LDL-receptor (4, 125).

In 2002 Scarselli *et al* (165), proposed a third receptor and mechanism of entry for HCV that may play a role in the tissue-tropism of the virus. Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (CD209) is expressed to high levels on endothelial cells of the liver and has been shown to induce the rapid internalization of HCV pseudo-particles after binding. It is currently thought that the binding of HCV to CD209 is mediated through the E2 protein similar to CD81. Further work by Lozach *et. al.* (103) could not reproduce the results showing the rapid internalization of the HCV sudo-particles after binding.

The current model for HCV infection has been significantly modified since the identification of a tissue culture adapted virus. HCV entry is now believed to be a multistep process involving four different viral receptors. The virion initially attaches to heparin sulfate along with the low-density lipoprotein receptor. Following this initial interaction the virus binds CD81 and SR-B1 which triggers the viral uptake via Claudin-1 and occludin (25).

Overview of CD163

CD163, originally termed M130 and RM3/1, was identified in 1987 as a molecule that was capable of producing an anti-inflammatory response (92, 220). It is a 130-kDa protein that consists of 9 scavenger rich-cystine rich (SRCR) domains, a transmembrane element, and a short cytoplasmic tail. There have been multiple splice variants of the protein identified in various species including both human and porcine (73, 74). CD163 has since been defined as a member of the scavenger rich-cystine rich superfamily due to its structural and organizational characteristics. The expression of CD163 is restricted to cells of the monocyte-macrophage lineage.

Originally, when researchers were looking for the functional role of CD163, it was believed to take part in either phagocytosis or the local immune regulation. To further elicit information about these proposed roles of CD163 the monoclonal antibody EDHU1-Ab was used to preincubate cells before phagocytosis experiments were conducted. No difference in the phagocytosis of either *S. aureus* or *E. coli* were observed after preincubation with EDHU1-Ab. While a complete understanding of the cellular functions has not been reached, it is now believed

to play a role in haptaglobin/hemoglobin scavenging (53). This receptor is upregulated in the presence of anti-inflammatory cytokines such as IL-10 and the soluble version of CD163 is believed to play a role in the anti-inflammatory response (156). Currently PRRSV and African Swine Fever Virus (ASFV) are the only viruses that have been identified using CD163 as a viral receptor (29, 162).

It has been demonstrated that it is possible to modulate the overall expression levels of CD163 on the cell surface of macrophages by treatment with a number of different molecules including 12-O-tetradecanoylphorbol-13-acetate (TPA), IL-10, and lipopolysaccharide (LPS). Macrophages and monocytes respond differently to these stimuli. CD163 expression on monocytes undergoing differentiation can be modulated. For example the combined treatment of monocytes with IL-10 and GM-CSF increased CD163 expression (186). While the treatment of monocytes with GM-CSF and TPA or LPS decreased expression of CD163. In contrast treatment of macrophages with IL-10 leads to no significant upregulation, while TPA caused a marked decrease in CD163 levels (26, 186).

Viral Genome and Replication

How RNA Viruses Replicate in Host Cells

The positive sense genome alone is all the viral material that is required to start the viral replication cycle. Once the virus has entered the cell, escaped from the early endosome, and fully uncoated. The hosts ribosomes can directly recognize the single stranded positive sense RNA as an mRNA. The positive sense RNA is either translated into one large polyprotein or multiple smaller proteins that are encoded within the viral genome (75, 179). The genome of

both HCV and PRRSV encode one or two large initial polyproteins respectively (120, 211). For

PRRSV these polyproteins encode all of the nonstructural proteins including the RdRp and

proteases that are needed to generate the subgenomic mRNAs and replicate the viral genome.

The required proteases are encoded within the initial polyprotein. These proteases are involved

in the initial cleavge of the large poly protein into all the constituent proteins. A hallmark of the

Arteriviridae family is the incorporation of a series of subgenomic RNA's that each encode a

different structural protein (20, 128, 164). For HCV this one large polyprotein includes both the

structural and nonstructural proteins that are needed for further viral replication (50, 101, 157,

210).

PRRSV Virus Replication

The PRRS virus is a member of the *Arteriviridae* family, which along with the *Coronaviridae*

and *Roniviridae* families comprises the genus *Nidovirales*. PRRSV is a single stranded positive

sense RNA virus with a genome size of approximately 15 kb, the exact size is strain dependent

(38). The viral genome encodes 8 open reading frames (ORF) which encode at least 22 viral

proteins (Figure 1-1B).

The PRRSV glycoproteins GP2a, GP2b, GP4, and GP5 are all actively involved in the binding of

the PRRS virion to the target host cell. GP2 encodes two open reading frames GP2a and GP2b

(175). GP2a contains two distinctive hydrophobic peaks in both the European and North

American Strains of PRRSV supporting two separate N-linked glycosylation sites (115, 121,

122, 129). The small ORF2 protein has been repeatedly demonstrated to associate with

intracellular membranes both during active PRRSV replication and after over expression of GP2b (Figure 1-1A) (121).

GP4 is a minor membrane glycoprotein that has been associated with neutralizing antibodies after Lelystad infection, however these neutralizing epitopes located in the amino terminal amino acids 40-79 are not conserved within the North American strains of PRRSV (119, 197). GP4 has a mass ranging from 20 to 35 kDa depending on the level of additional N-glycans that are obtained during the transport through the ER and Golgi (119). GP4 is an immunogenic protein with approximately 65% of PRRSV-positive sera from swine farms within the US and Canada reacting to recombinant GP4. GP4 contains multiple hydrophobic sequences at both ends of the protein as well as four N-linked glycosylation sites that are conserved across all strains (111, 115, 122, 123). It has been suggested that GP4 is involved with the binding of CD163 and internalization of the virion into the cell.

ORF5 encodes GP5, the major envelope glycoprotein in the PRRS virion, with an estimated weight between 24.5 and 26 kDa (110, 111, 122). There is an N-terminal signal sequence and multiple glycosylation sites. There is significant genetic variation within GP5 between the Lelystad like viruses and their North American counterparts, but the hydropathy profiles remain similar (111, 115, 130). The GP5 protein from both the North American and European strains has been shown to have an apoptotic effect in both monkey kidney cells and PAMs (180, 181). The over expression of the anti-apoptotic protein BCL-2 did not prevent the GP5 induced apoptosis of these cells (180). The GP5 protein is know to form a heterodimer with the PRRSV

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M protein creating the primary receptor binding complex (110, 190). Antibodies to GP5 can neutralize PRRSV (197).

The M protein is encoded for by ORF6 and has an estimated mass between 18 to 19 kDa. It possesses three hydrophobic regions assumed to be membrane spanning domains similar to the coronavirus M protein and is non-glycosylated (111, 123).   The M protein forms disulfide linked heterodimers with GP5 in the ER before further transport of these protein occurs, absence of these disulfide bonds prevents translocation to the golgi (110). The M protein is believed to play a role in viral assembly and budding (110).

The nucleocapsid (N) protein is 123 AA, NA strain, or 128 AA, European Strain, in size with a molecular weight between 14 and 15 kDa. It is a highly basic protein that accounts for between 20 and 40 percent of the total protein in the PRRS virion (12, 109, 111, 122, 134). The N protein is present primarily as a homodimer and multimerizes to form the icosahedral core protein of the PRRS virus (110, 122). There are multiple conserved immunogenic epitopes between the North American and European strains of this virus (42, 47, 118, 134).

The GP5 and M heterodimer comprise the main membrane proteins of the PRRSV virus which are responsible for the initial binding of the virion to the host cell (43, 82, 205). Recent research suggest that GP5 and M heterodimer bind sialoadhesion on the cell surface and then enters the target cell via clathrin-mediated endocytosis facilitated by the interaction of the viral GP2a and GP4 with CD163 (41, 187-189). The PRRS virus then moves into the cell via the early endosome where the viral RNA is released into the cell (188). After uncoating in the early

endosome and the release of viral RNA into the target cell ORF 1a and 1ab are initially

translated (Figure 1-1C). The two initial polyproteins are further cleaved by a series of four

different proteases including papin-like cysteine proteases PCPα and β located within Nsp1, a

cysteine like protease located within Nsp2, and the main serine protease 3CL Pro that is located

within Nsp4 (5, 7, 19, 176, 219).

The second step is the transcription of negative sense and subgenomic RNA fragments. After the

expression and cleavage of these non-structural proteins, these proteins interact with the viral

genome transcribing the minus-strand subgenomic RNA's and full genomic template from the 3'

towards the 5' end of the viral genome (116). ORF's 2 through 7 encode all of the structural

proteins including: GP2a, GP2b, GP3, GP4, E, M, and N. These proteins are expressed from a

nested set of subgenomic RNA's (43, 117, 120, 203, 204). Each separate subgenomic ORF is

preceded by a short leader sequence that is recognized by the 5' leader sequence. The synthesis

of the minus-strand is discontinuous and yields a series of minus-strand subgenomic RNA's that

are used as template for the generation of plus-strand subgenomic RNA's (20, 128, 133, 144).

The positive sense subgenomic RNA's are then translated into the proteins corresponding to

ORFs 2-7 (164).

Hepatitis C Virus Replication

The HCV viruses are divided into 6 genotypes and multiple subtypes and are a member of the

Hepacivirus genus and Flaviviridae family (50, 83, 85, 101). Similar to other members of the

Flaviviridae family it is surrounded by a lipid bilayer that is anchored by multiple envelope

proteins (Figure 1-2A). This envelope envelopes the nucleocapsid core that is composed of the

HCV RNA genome and the core protein. The HCV virus is composed of a positive sense single

stranded RNA genome that encodes a single large polyprotein. The gnome is 9.6kBp encoding

over 3000 amino acids (Figure 1-2B) (1). The polyprotein encodes at least 10 distinct proteins

and is cleaved by the NS3-4a protein (16, 40, 112). The majority of the structural proteins are

encoded on the 3' end of the viral genome. The main open reading frame within HCV is flanked

on either side by both a 5' and 3' untranslated region.

The length of the untranslated regions within this family of viruses is variable ranging from 95-

555 bp for the 5' UTR and 114-624 bp for the 3'UTR. The 5' UTR of HCV contains 341 nt

prior to the beginning of the main ORF (22, 30, 68, 76, 170, 177). Within this ORF there are 4

conserved regions corresponding to 4 separate highly structured domains. The domains 2

through 4 and the 5' most portion of the HCV core protein form the HCV internal ribosome

binding site which is capable of directly binding the 40s ribosomal subunit (138, 185, 193, 194).

Similar to the 5' UTR the 3' UTR contains multiple stem loop structures and is divided into three

distinct regions. The first region consists of a 30-40 nt variable region directly followed by a

poly(U)-poly(UC) stretch. The final region is a highly conserved stretch encoding 3 stem-loop

structures termed SL1, SL2, and SL3 (68). The 3' UTR has also been shown to interact with the

HCV RdRp, NS5b, through multiple stem loop structures (55). Any disruption in the 52 nt

upstream of the poly(U) tract or the multiple stem loops results in the disruption of RNA

replication

The HCV virion is comprised of 3 main structural proteins the two envelope proteins and the

core protein. The E1 and E2 proteins are responsible for the initial binding of the HCV virion to

CD81 and are essential for the viral life cycle. These two proteins are responsible for numerous other viral functions including membrane anchoring, ER localization, and heterodimer assembly (141). Both the E1 and E2 proteins are transmembrane glycoproteins that contain 5 or 11 glycosylation sites respectively (51). The E2 protein contains a region often termed hyper-variable region one. This region can differ in sequence homology by up to 80% depending on the genotype of HCV virus (191). Although there is high nucleotide variability in this region the overall protein structure, and biochemical properties of each of the amino acids remain constant suggesting this region is crucial to the viral lifecycle (146). This hyper-variable region consists of 27aa and is a major neutralizing epitope (148).

At the center of the HCV virion is the core protein which comprises the other structural protein encoded for in the HCV genome. This protein is a highly basic protein that has been shown to bind RNA and is thought to play a major role in the formation of the HCV capsid. Upon original processing the core protein is 191 aa and approximately 23kDa. The final form of this protein appears to be predominantly 21kDa although sizes ranging from 17 to 23 kDa have been detected (171). This protein consists of three separate domains consisting of and N-terminal hydrophilic region, a C-terminal hydrophobic region and a short signal peptide located on the 3' end of the protein (84, 147). The positive charges found within the N-terminal domain are often characteristic of the RNA binding portion of a protein and also contains multiple nuclear localization sequences (80). The core protein has also been shown to have several ties to the viral life cycle including interaction with cellular proteins and both pro and anti-apoptotic properties (31, 100, 137, 209).

After the initial binding of the virion and internalization via claudin-1 and occluding into the

early endosome the membrane of the HCV virion fuses with the endosome. This fusion allows

the viral core to escape into the hosts cytoplasm where the 40s subunit directly binds to the IRES

within the HCV genome. After the binding of the ribosome to the IRES one large polyproteins is

initially produced. The NS3-4a protease plays a crucial role in the proteolytic cleavage of the

large HCV polyprotein. Multiple groups have determined the structure of the NS3-4a protein

and the catalytic triad has been localized to His 57, Asp 81, and Ser 139 (67). NS3 alone also

contains a domain termed the NS3 helicase-NTpase domain. Multiple functions of this domain

have been identified including RNA-stimulated NTPase activity, RNA binding, and unwinding

of secondary and tertiary structures of RNA. Levin *et al* (94) suggested that the NS3 helicase

translocates along the RNA (94). Recently other functions of the NS3-4a complex have recently

been elucidated. This complex has been implicated in the regulation of the innate immune

system during viral infection. In 2003 the NS3-4a protein was shown to antagonize the dsRNA-

dependent interferon regulatory factor 3 (IRF-3). IRF-3 is a critical player in the modulation of

the interferon induction in response to viral infection (63, 96). This does not appear to be the

only location in this pathway that NS3-4a disrupts. Further studies in 2005 suggest that NS3-4a

blocks the signaling of dsRNA through the TLR-3 receptor (97). In addition to the disruption of

the IFN system in response to viral infection the viral protease is also responsible for the

cleavage of the large precursor polyprotein (Figure 1-2C).

The other nonstructural proteins that participate in the formation of the replication complex and

modulation of various cellular pathways include NS4b, NS5a, and NS5B. NS4b appears to

function as a membrane anchor for the HCV replication complex. Structural predictions indicate

that there may be up to four distinct transmembrane domains included within this 241 aa protein which may play a role in the ER membrane localization (98).

NS5a is crucial for HCV replication and is found in association with lipid rafts (113). It has been implicated in the modulation of various cellular pathways and known to interact with the vesicle-associated membrane-associated protein. There is currently evidence that NS5a hyperphosphorylation can cause the negative regulation of HCV RNA synthesis by disruption of hVAP-33 (65). Further reports suggest that NS5A hyperphosphorylation is crucial in the virus lifecycle because it acts as the trigger that pushes the virus from a replication stage into an assembly stage (78). Direct interaction between NS5A and NS5B have been observed but the actual effects of this interaction are currently unknown (99).

NS5B is the RNA-dependent RNA-polymerase encoded within the virus (14). NS5B has recently been reported to bind to cyclophillin B which appears to regulate HCV replication (195). NS5B has been reported to be a tail-anchored protein with the terminal 21 residues anchoring to the ER exposing the proteins functional domain (167). Structural analysis of NS5B has shown that it has a classic RdRp structure (108). Each of these structural and nonstructural proteins plays a crucial role in the replication of the HCV genome, alteration of cellular pathways, and blocking of innate immune responses.

Viral Interactions with the Host Cell

All viruses require a number of cellular factors for their replication. This reliance on these various host factors has directly tied the replication cycle of many viruses to specific pathways

within their host cells. Disruption of these individual pathways is known to reduce the viral replication levels. Viruses such as Dengue and West Nile Virus have demonstrated the ability to modulate their respective host cell cycles. Synchronization of the cell cycle to a stage other than that required by the virus greatly reduces the overall viral levels (33). Measles virus induces a cell cycle hold at G0, which is believed to play a role in the virus's immunosuppressive abilities (132).

Multiple viruses including influenza, PRRSV, vaccinia virus, and HCV have developed ways to disrupt the innate immune response in their host cells. Influenza's NS1 can bind dsRNA and sequester retinoic acid-inducible gene 1 (RIG-1) preventing the downstream activation of interferon response factor-3 (IRF-3). The PRRSV NS1 protein has recently been shown to attenuate IRF-3 and NF-kappaB-dependent gene induction by dsRNA (17). Vaccinia virus encodes over 200 individual genes, over half of which are not necessary for viral replication in cell culture. The majority of these genes encode proteins that are responsible for the modulation of various host cell pathways, the innate immune response, or have some function in the host infection cycle.

The ERK Pathway

The ERK pathway is stimulated by multiple factors including growth factors and bile acids. The binding of one of these stimulants to the cell surface receptor EGFR initiates signaling through a series of tyrosine kinases. EGFR contains 9 separate phosphorylation sites, 8 of which are autophosphorylated (95). These phosphorylated sites function as the binding site for the Shc homology 2 and phosphotyrosine binding domains of multiple adapter proteins (166). After the

binding of growth factor to EGFR the signaling pathway requires the presence of Shc, Grb2, and

SOS to initiate the phosphorylation of RAS. RAS is a member of the GTPase family of proteins

that hydrolyze GTP and was first identified as a transforming oncogene of the murine sarcoma

viruses. Viruses have been shown to encode three separate forms of RAS Harvey-Ras (H-Ras),

Kirsten-Ras (K-Ras), and Neuroblastoma-Ras (N-Ras) (173). These Ras variants all lack the

ability to hydrolyze GTP and are constitutively active. Ras activates the next step in the pathway

Raf.

Raf was originally discovered as a viral encoded transforming agent v-raf (154). Raf is a

member of the MAP3K family and is classified as a Ser/Thr kinase. Ser/Thr kinases catalyze the

phosphorylation of both Ser and Thr residues. Mammals are known to express 3 different forms

of Raf ranging in size from 70 to 100 kDa (93). To fully activate Raf 4 separate phosphorylation

sites must be phosphorylated by Ras. Of the multiple isoforms of Ras that have been identified

they all activate Raf with varying efficiencies (32). V-Ras has the highest activation efficiency

of Raf that has been identified. After the activation of Raf, Raf further activates MEK (208).

MEK 1 and MEK 2 are alternatively known as MKK1 and MKK2. Raf activates MEK by the

phosphorylation of multiple serine residues located at positions 217 and 221 (6). Unlike Raf

MEK is found at high levels within the cells. This allows for the rapid amplification of the

signaling pathway after the initial activation events (77). No viral encoded mutants of MEK

have ever been identified, although when MEK is constitutively activated it has been shown to

be tumorgenic (107).

ERK is the heart of the pathway that leads to the activation of a number of downstream events. ERK1 and 2 are expressed in all examined mammalian tissues with ERK2 being expressed to slightly higher levels (142). For ERK to be fully activated it requires the phosphorylation of both Thr202 and Tyr204 for ERK1 and similarly Thr185 and Tyr187 for ERK2 (56). For total control over the ERK pathway there are 9 known dual specificity phosphotases that can terminate ERK activation. The ERK pathway is a critical regulator of the expression of multiple genes associated with cell cycle regulation. The activated form of ERK is responsible for the regulation of growth factor-response targets located in the cytosol. As well as the activation of these growth factor-response targets it also translocates to the nucleus where it controls the phosphorylation of multiple transcription factors that regulate gene expression (24).

Viruses Affected by Alteration of the ERK Pathway

The replication of multiple viruses has been tied to the ERK pathway. Herpes Simplex virus type 2 has been shown to cause a sustained activation of ERK1/2. When MEK1/2 are knocked down using siRNA or MEK activation is attenuated by treatment with U0126 the replication of Herpes Simplex virus type 2 is greatly reduced (216). Kaposi's sarcoma-associated herpes virus (KSHV) has also been shown to activate both MEK and ERK during the course of infection. When the host cells were treated with U0126 viral DNA internalization was still observed but nuclear delivery of KSHV DNA was significantly attenuated. Analysis of gene expression after treatment with U0126 showed the expression of multiple genes was severely attenuated and only a limited viral infection was observed (172). During reovirus infection the viral oncogene CUG2 enhances viral replication through the activation of both Ras and p38 signaling (143). Hepatitis

B virus controls the regulation of the c-met promoter through the activation of ERK and

treatment of HepG2 cells with U0126 can significantly attenuate viral replication levels (206). A

number of paramyxoviruses also alter the expression and activation of both ERK1/2. Inhibition

of the ERK pathway with U0126 completely eliminates expression of viral proteins after

infection (28). Bovine viral diarrhea virus (BVDV) infection in MDBK cells results in the

increased activation of ERK1/2. It was shown that BVDV affects the ERK signaling though

oxidative stress (207). These data show that multiple viruses have the ability to alter the

activation levels of the ERK pathway and that attenuation of this activation can significantly

reduce the overall course of viral infection.

How the ERK Pathway May Play a Role in HCV Replication

HCV protein NS5A alters the trafficking of EGFR and the activation of the ERK pathway.

Attenuation of signaling through the ERK pathway has recently been demonstrated to reduce the

overall levels of HCV RNA. Scholtes *et al*. also demonstrated that the treatment of HUH7 cells

with a MEK1 inhibitor U0126 can prevent the replication of HCV after infections (168). Further

studies have identified that the enhancement of the ERK pathway can enhance the overall levels

of HCV replication (23). It is currently unknown why HCV depresses the activation of the ERK

pathway but it has been suggested that NS5A may play a role in the creation of a persistent HCV

infection (155).

Model Systems for Study of RNA Viruses

When PRRSV was originally identified it was only able to be cultured on porcine alveolar macrophages (PAMs). Work to find a separate suitable immortal cell line lead to the identification of MARC-145 a sub clone of MA-104 cells (87). Infection of confluent monolayer's of MA-104 cells showed limited infection of the PRRSV virus with no significant viral production. This suggested that a subpopulation of these cells were capable of supporting replication of the PRRSV. The original MA-104 stock subcloned to identify a more permissive MA-104 clone, this clone was termed MARC-145. MARC-145 cells show a high susceptibility to the PRRSV virus with titers often reaching 10^7 log $TCID_{50}$ (88).

HCV

The genome of HCV was first cloned in 1989, but until recently there had been limited success in isolating a HCV virus that could be successfully tissue culture adapted (34). This lack of any tissue culture adapted viruses created the need for a secondary system for the study of the HCV virus. The 5' end of the viral genome consists of a non-translated region (NTR) that acts an internal ribosomal entry site (IRES) (182). The genome for the first replicon-harboring cells was isolated from an infected human liver and recovered using long distance RT-PCR. A T7 polymerase promoter and an engineered restriction site on the 3' end of the clone allowed for the efficient production of RNA transcripts. When transfected these initial RNA transcripts showed no evidence of replication in any of the cell lines they were tested in. To solve this problem constructs were generated encoding G418 to enable the selection of cells supporting HCV replication. The HCV RNA within these cells replicated to high titers and expressed all of the encoded viral genes (102). The genomic organization of the current group of replicon-harboring cells is slightly modified from the wild type genome. The 5' NTR directs the translation of the

neomycin phosphotransferase gene. This is followed by the IRES of the *Encephalomyocarditis virus* (EMCV) which directs the translation of the encoded HCV regions (90). There currently exist replicon-harboring cells from the majority of HCV genotypes. Replicon-harboring cells possess a number of advantages when testing the effectiveness of various antiviral compounds. They allow for the rapid screening of multiple compounds across a broad array of HCV genotypes. The effectiveness can be rapidly quantified using qRT-PCR and supplemented with western blot analysis. The first HCV virus to be tissue culture adapted was the genotype 2 JFH strain (101). This was followed by the isolation of a genotype 1 virus by Yi *et al.* (212) in 2006 that allowed for the recovery of infectious virus particles. These are the first viral systems that allow for the analysis study of a productive HCV infection *in vivo.*

Significance of HCV and PRRSV

PRRSV Significance

The PRRS virus causes significant damage throughout all phases of swine production. In adult swine infection with the PRRS virus causes reduced appetite, fever, premature farrowing and abortions, death in up to 10% of sows, and in extreme cases neurological symptoms. When pregnant sows are acutely infected with PRRSV the affected litters can be inflicted with a number of different problems, ranging from still born pigs, high pre-weaning mortality, mummified pigs, variable sized weak pigs, and adema around the eyes. Within the group of weaned pigs infection results in loss of appetite, lethargy, failure to thrive, labored and rapid breathing, blotchy or reddening skin, and a distinctly rough hair coat. This virus has been shown to cause persistent infection in animals and has been detected in boar semen at greater than 200 days post infection (35, 36, 174).

The wide range of problems associated with the PRRS virus costs the US swine industry an estimated 600 to 700 million dollars a year causing a significant impact on the overall economic stability of the swine industry (136). Due to the lack of an effective vaccine many of the protocols involved in the elimination of PRRSV from production barns can involve a costly depopulation/repopulation strategy. These strategies often involve the barn sitting empty for a significant period of time to ensure that all virus has been eliminated from the barn. There have been some successful attempts at the eradication of PRRSV from specific areas. Following detailed biosecurity protocols helps to prevent the spread of PRRSV but there is current research suggesting that PRRSV can be transmitted limited distances through aerosol (44).

Viral spread throughout a swine barn can take place within a period as short as 7-10 days. This rapid spread can make it difficult to control an outbreak due to the fact that by the time the symptoms are noted in the originally infected animals a large portion of the barn is potentially infected.

HCV Significance

HCV is a global health problem with the virus chronically infecting over 170 million people worldwide. The estimated cost of treatment for the 4.5 million infected patients in the United States that develop chronic liver disease as a result of HCV infection is expected to reach 360 billion dollars. The average lifetime treatment for an HCV patient who does not develop chronic liver disease is $100.000 dollars over their lifetime. Nearly 1,000 patients a year receive liver transplants yearly due to the effects of HCV infection. In the current economy a liver transplant

costs approximately $280,000 dollars. This amounts to nearly 300 million dollars a year alone in

financial losses. The net result is a major financial impact in the United States alone, these

number do not estimate any of the medical costs from other nations (9, 81, 178).

Control of Viral Outbreaks and Spreading

Many different approaches have been taken to control the spread of virus's including vaccination

and the use of antiviral drugs. Unfortunately there exist a number of RNA viruses that are

currently no vaccinations for and our current antiviral therapies either are not effective or are a

long and drawn out sometimes painful process. Viruses have adapted a number of effective

strategies for evading the host immune system. Viral adaptations vary dependent on the virus,

some viruses have adapted mechanisms create persistent infections hiding from the hosts

immune system, and others posses the ability completely overwhelm both the innate and

adaptive immune system of their host. Several viral infections are actually enhanced by out

immune system through a form of antibody mediated enhancement.

Within the replication cycle of each virus there are several different points of attack when trying

to control the replication of these pathogens. These begin with the viral receptor and the initial

entry into the host cell, followed by uncoating, replication, assembly, and finally maturation and

release. Often times many cellular pathways play an active role in the replication of these

viruses and also offer an attractive target for antiviral development. In this series of studies we

modulated the expression level of the PRRS viral receptor CD163, and explored the interactions

between HCV replication, the ERK pathway, bile acids, and multiples small molecule antiviral

compounds.

Control of Hepatitis C Virus

HCV is commonly spread through a variety of means including blood transfusions, reuse of needles, and sexual intercourse. The control of HCV within a population begins with education. Many routes of HCV transmission can be easily controlled including the use of protective measures during sexual intercourse, always using disposable needles, and the screening of all blood meant for transfusion purposes.

The current method of treatment using long acting pegylated interferon-α and ribavirin are the only effective measures available to our medical practitioners today. The development of vaccines and antivirals has long been hampered by the lack of any tissue culture adapted viruses. This forced researchers to rely on replicon-harboring cells based upon either the full length HCV genome or smaller subgenomic replicon systems (102). Recently both genotype I and II viruses have been isolated that were successfully tissue culture adapted giving researchers there first chance to work with a replication competent HCV viral system (101, 212).

A number of antiviral compounds have been identified against HCV through the use of replicon-harboring cells, and there are currently a number of antiviral compounds being evaluated for their efficacy against HCV both in the lab and in clinical trials (37, 58, 104, 131). Groups of specifically targeted antiviral therapies against HCV include antivirals specifically targeted against two main proteins within HCV: NS5B the RNA-dependent RNA-polymerase, and NS3 the viral protease. The rapid mutation rate of the HCV genome has currently prevented the development of a viral specific antiviral that the virus is unable to escape. The escape potential of HCV requires the continuous development of new antivirals. A second group of HCV

antivirals based upon the inhibition of cellular factors that are essential to viral replication are also being developed. The prototypical member of this family is cyclosporine A (58, 131). Cyclosporine A has been shown both *in vivo* and *in vitro* studies to significantly reduce the overall HCV levels. The downside of Cyclosporine A is its immunosuppressive effects. Currently a number of non immunosuppressive derivatives including Debio-25 and NIM811 have been synthesized and are being evaluated for their anti-HCV potential in various clinical trials (37, 104).

Figure 1-1. The PRRSV Virion.

A) The Structure of the PRRS Virion B) Genomic Organization of PRRSV C) Replication of

PRRSV
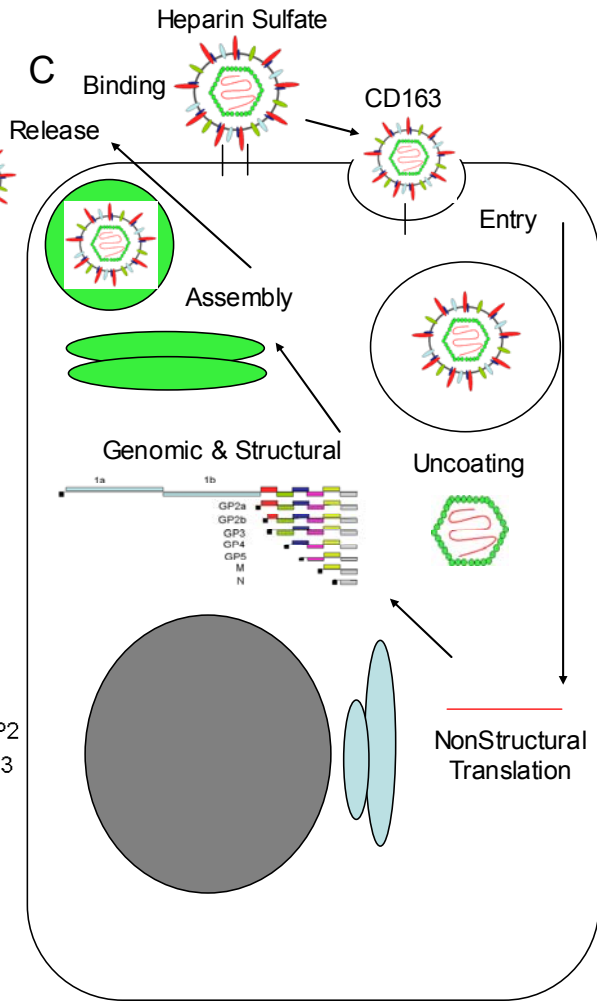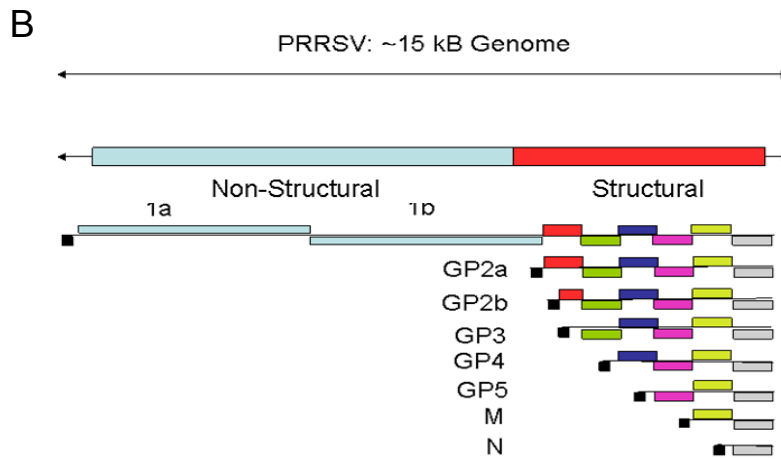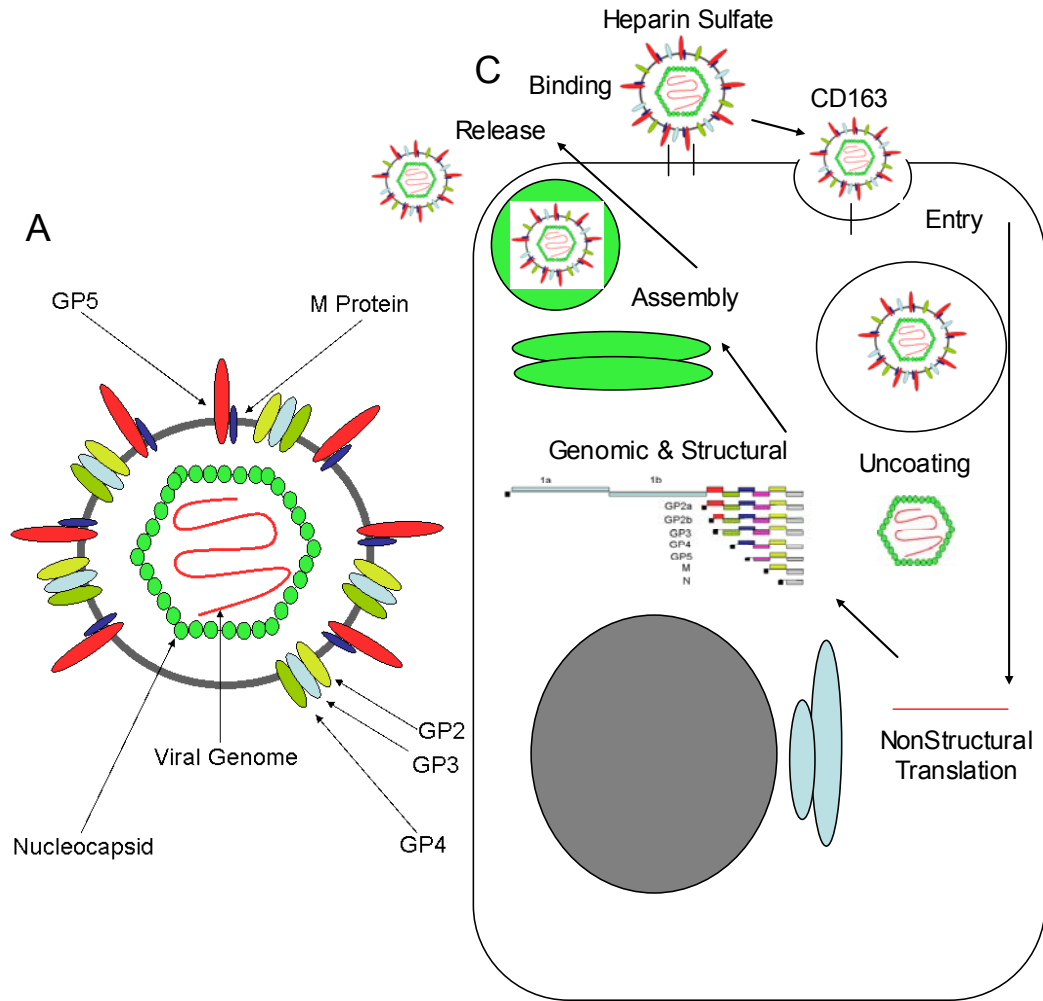
Figure 1-2. The HCV Virion.

A) The structure of the HCV virion. B) Genomic organization of HCV C) Replication of HCV

within the host cell.
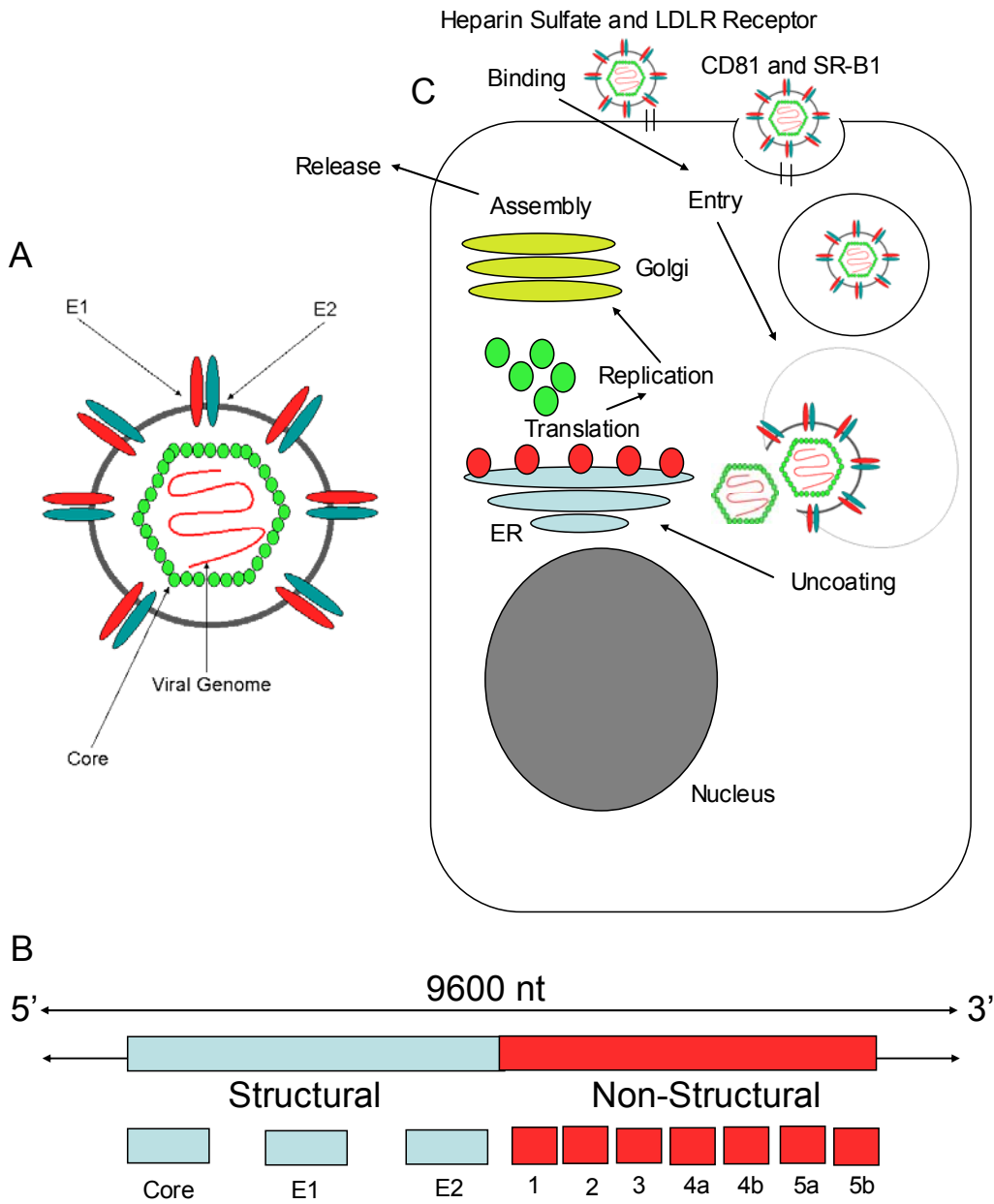
Figure 1-3. The ERK Pathway.
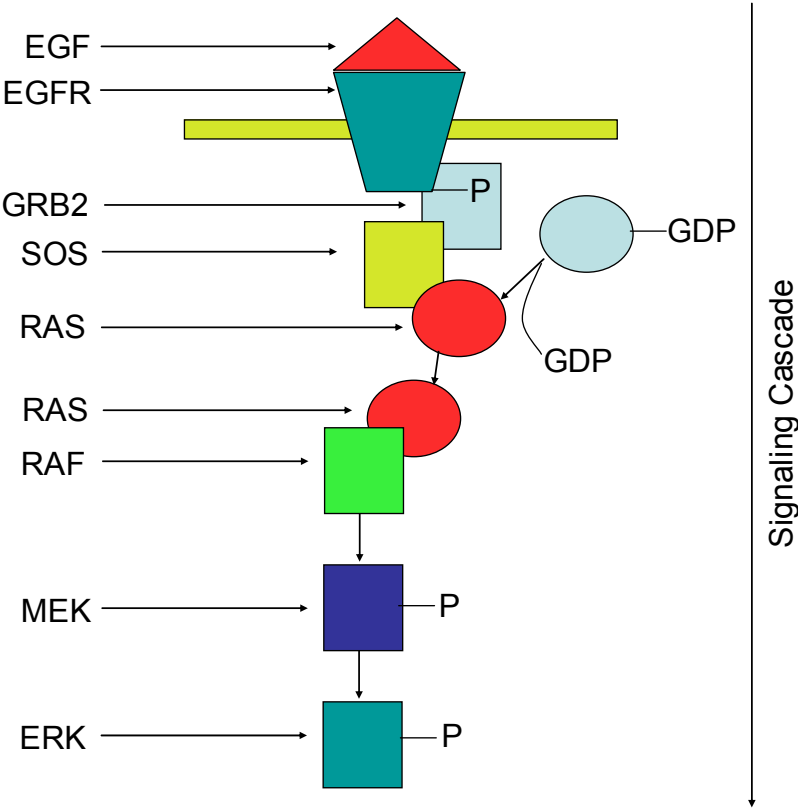
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CHAPTER 2 - Modulation of CD163 Receptor Expression and Replication of Porcine Reproductive and Respiratory Syndrome Virus in Porcine Macrophages

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) has a specific cell tropism for differentiated macrophages, such as porcine alveolar macrophages (PAMs).We analyzed the expression of CD163 on PAMs and macrophages derived from CD14 positive blood monocytes (MDMs), in correlation with PRRSV replication. By flow cytometry analysis, we showed that the levels of CD163 expression correlated well with the overall level of PRRSV replication. We further examined the effects of modulators of macrophage function, including 12-O-tetradecanoylphorbol-13-acetate (TPA), lipopolysaccharide (LPS), and interleukin (IL)-10 on the expression of CD163 and PRRSV replication. Pre-treatment of PAMs or MDMs with TPA or LPS resulted in decreased expression of CD163 and reduction in PRRSV replication. On the contrary, the incubation of CD14 positive monocytes with IL-10 during differentiation into MDMs resulted in up-regulated expression of CD163 with a corresponding increase in PRRSV infection. These data indicate that the expression of CD163 on macrophages in different microenvironments, in vivo, may determine the replication efficiency and subsequent pathogenecity of PRRSV.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), caused by PRRS virus (PRRSV), was first described in the United States in 1987 (23) and a few years later in the Netherlands (52).

PRRSV is a member of the family *Arteriviridae*, which is composed of a group of positive sense, single stranded RNA viruses, including simian hemorrhagic fever virus (SHFV), equine arteritis virus (EAV), and lactate dehydrogenase-elevating virus (LDV) (5, 6, 28, 29, 55). At present, 2 genotypes of PRRSV have been described: European (Type I), and North American (Type II). Sequence analysis has shown approximately a 60% nucleotide identity exists between the Type I and Type II genotypes (16, 20, 31, 35). Both genotypes have since been identified as a major viral pathogen in swine causing reproductive failure, respiratory distress, and a decrease in average weight gain, and are responsible for over 560 million dollars in losses per year in the United States alone (33). The PRRSV virion contains a genome of approximately 15 kb in length that encodes at least nine open reading frames (6). The genome is completed with the addition of a 5' cap and 3' poly(A) tail (29). The major and minor structural proteins are encoded by a nested set of subgenomic RNAs located on the 3' end of the genome. The major structural proteins consist of the nucleocapsid (N) and membrane protein (M) as well as the major envelope glycoprotein (GP5), and the minor structural proteins include GP2a, GP2b, GP3, and GP4 (8, 54).

PRRSV has a tropism for cells of the monocytic lineage, especially differentiated macrophages such as porcine alveolar macrophages (PAMs) (21). PRRSV primarily replicates in PAMs, but it has been identified in macrophages located in tissues including spleen, liver, Peyer's patches, and thymus (26, 27, 37). PRRSV isolates can be serially passaged in vitro in immortal cell lines derived from MA104 (African green monkey kidney) cells, including MARC-145 (1, 24). However, there remain gaps in our knowledge regarding the factors that regulate cell tropism. Several receptors for PRRSV have been described, including heparin sulfate, sialoadhesin, and

vimentin (9, 10, 24). Recently, CD163, a molecule that is expressed exclusively on cells of a monocytic lineage, has been identified as a possible cellular receptor for PRRSV (4). Calvert *et al.* (2007) identified CD163 as a receptor that allowed previously nonpermissible cells to become susceptible to PRRSV infection. CD163 is a hapten/hemaglobin scavenger receptor in the scavenger receptor cysteine-rich (SRCR) superfamily (38, 40, 53). CD163 is currently thought to be involved in anti-inflammatory responses and the removal of haptoglobin and hemoglobin from the blood (17). The expression of CD163 on macrophages is inducible and can be either up- or down-regulated by treatment with different stimuli including interleukin (IL)-10, dexamethasone, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4 (2). In addition to the surface expression of receptor and co-receptor molecules, other factors appear to be necessary for PRRSV permissiveness (3).

In this report, we provide further evidence for CD163 being a PRRSV receptor. First, we demonstrate that a non-permissive cell line, LLC-PK, can be made permissive for PRRSV replication by expression of a full-length porcine CD163. Second, we found that an anti-CD163 monoclonal antibody could reduce the infection levels of PRRSV in PAMs. Third, we show that surface expression of CD163 on PAMs and CD14 positive monocyte-derived macrophages (MDMs) can be modulated following treatment with IL-10, LPS, or TPA. Finally, the expression level of CD163 correlates with the level of PRRSV infection. These data suggest that the expression of CD163 on macrophages in different microenvironments in vivo possibly may determine the replication levels of PRRSV and the virus pathogenicity.

Materials and Methods

Cells and Reagents

MARC-145 and LLC-PK cells were maintained in Eagle's minimal essential medium (EMEM, Mediatech, Herndon, VA) containing 5% fetal bovine serum and antibiotics [penicillin (250 U/ml) and streptomycin (250 μg/ml)]. PAMs and MDMs were maintained in RPMI-1640 (Mediatech) supplemented with 10% fetal bovine serum (FBS) and antibiotics. 12-O-Tetradecanoylphorbol-13-acetate (TPA) and lipopolysaccharide (LPS) were obtained from Sigma–Aldrich (St. Louis, MO). TPA or LPS was reconstituted in DMSO or distilled water at 50 mM or 10 mg/ml, respectively. Recombinant IL-10 and GMCSF were obtained from R&D systems (Minneapolis, MN) and was reconstituted in PBS. An anti-PRRSV nucleocapsid antibody conjugated with FITC (SDOW17) was obtained from South Dakota State University (Brookings, SD). Antibodies to porcine CD14 and CD163 were purchased from AbD Serotech (Raleigh, NC). PRRSV strains, SD01-08 (SDSU, Type I) (12, 13) and P129 (Type II, isolated and characterized at the Animal Disease Diagnostic laboratory, Purdue, West Lafayette, IN, Calvert *et al.*, 2002 [GenBank Accession #AF494042]) were maintained by passaging in MARC-145 cells. The PRRSV expressing GFP (129-GFP) was generated by transfecting the Kermit (Yoo *et al.*, 2004) infectious clone (Pfizer Animal Health, Kalamazoo, MI) in MARC-145 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Virus stocks were prepared and titered in MARC-145 cells and stored in aliquots at −80°C until use.

Generation of Virus Stocks

All virus isolates were cultured on 2–3-day-old confluent layers of MARC-145 cells in T-75 flasks. Virus was allowed to propagate until 70–80% CPE was observed and each flask was frozen and thawed 2 times to ensure cell lysis. Supernatant was then collected and aliquotted

before being stored at −80°C until use. Viral titers were determined by titration on MARC-145 cells. All experiments were carried out using a multiplicity of infection (MOI) of 1, as determined by titration on MARC-145 cells

Generation of LLC-PK Cells Constitutively Expressing CD163

The full length porcine CD163 was amplified from the total RNA of PAMs using RT-PCR with primers pCD163-Nhe-F (aattGCTAGCATGGACAAACTCAGAATGGTGC, Nhe1 restriction sequence underlined) and pCD163-Not-R (aattGCGGCCGCTCATTGTACTTCAGAGTGGTCTCC, Not1 restriction sequence underlined). Total RNA of PAMs was isolated using the RNeasy kit (Qiagen, Valencia, CA) and RT-PCR reactions were performed using the Titan RT-PCR kit (Roche, Indianapolis, IN). The amplified CD163 cDNA (~3.6 kb) was cloned into pIRES-Puro2 (Clonetech, Los Angeles, CA) using Nhe I and Not I restriction sites. After the construct was verified by sequence analysis, the plasmid was transfected into 1-day-old semi-confluent LLC-PK cells using Lipofectamine 2000 (Invitrogen). LLC-PK cells expressing porcine CD163 were selected using 4µg puromycin per ml of MEM containing 10% FBS. The media containing puromycin was replenished every 3–4 days during selection. After selection, individual cell clones were isolated in a limiting dilution method using 96 well plates, and the resulting colonies were screened for the expression of CD163 by immunofluorescent assay (IFA) using anti-porcine CD163. Several cell clones expressing high levels of CD163 were chosen and designated as LLC-PK-CD163. Further IFAs were performed using SDOW17 in the parental LLC-PK and LLC-PK CD163 cells to measure for PRRSV susceptibility using virus strains P129 and SD01-08. Two- or three-day-old confluent cells were inoculated with each PRRSV strain at a MOI of 1 and incubated for 24 h.

IFA staining was performed after the cells were fixed with 80% acetone. P129-GFP was also used to examine the infectivity of PRRSV in the cells by observing GFP expression on a fluorescent microscope.

Preparation of PAMs and MDMs Derived From CD14 Positive Cells

Porcine alveolar macrophages were collected from 7 to 8-weekold PRRSV negative pigs by lung lavage with phosphate buffered saline (PBS) during necropsy. Collected cells were washed 3 times in PBS and pelleted by centrifugation at 300×g for 10min. Cells were frozen at a concentration of $2×10^7$ cells/ml in liquid nitrogen until use. The frozen PAMs were rapidly thawed in a water bath (37^oC), washed in 10ml of PBS, and resuspended in RPMI containing 10% charcoal/dextran treated FBS (HyClone, Logan, Utah). The CD14 positive porcine monocytes were isolated from the whole blood of 7–8-week-old PRRSV negative pigs. The collected whole blood was overlayed on a ficoll gradient (Sigma–Aldrich) to isolate peripheral blood mononuclear cells (PBMC). The isolated PBMCs were stored at a concentration of $1×10^8$ cells/ml in liquid nitrogen. CD14 positive cells were then isolated from the PBMC using magnetic assisted cell sorting (MACS, Miltenyi Biotech, Auburn, CA) and a monoclonal antibody against porcine CD14. Briefly, cells (PBMC) were rapidly thawed and washed 2 times in PBS and collected by centrifugation at 300×g for 7 min. Cells were resuspended in 160μl of 2% FBS in PBS with 200mM EDTA (wash solution). Anti-porcine CD14 antibody (30μl) was then added and incubated on ice for 20 min. Cells were brought up in 10 ml of the wash solution and rinsed one time before collection at 300×g for 7 min. The antibody bound cells were resuspended in wash solution containing magnetic beads coated with anti-Mouse IgG (Miltenyi Biotech) and incubated on ice for 20 min. After incubation, cells were rinsed once in wash

solution, and applied onto a Millityec MACS sorter. CD14 positive cells were eluted in 1ml

RPMI-1640. The isolated CD14 positive monocytes were resuspended in RPMI-1640 containing

10% charcoal/dextran treated FBS and antibiotics. The CD14 positive cells were allowed to

differentiate into macrophages (MDMs) for up to 4 days in the presence of 10 ng/ml GM-CSF.

The Expression of CD163 and PRRSV Susceptibility in PAMs or MDMs

The population of PAMs or MDMs expressing CD163 at various stages were examined in

correlation with PRRSV infectivity. To accomplish this, freshly isolated (day 0) or 1–3-day-old

PAMs or MDMs were plated at 5×10^5 cells/well in a 6 well plate and inoculated with P129-GFP

at a MOI of 1. Virus infected cells were incubated for 18 h before the expression of CD163 or

GFP was measured by flow cytometry analysis as described below.

Treatment of anti-CD163 Monoclonal Antibody on PRRSV Infection in PAM

PAMs were incubated in dilutions of mouse anti-porcine CD163 antibody of 0, 1, 3, or 5

µg/100µl media in a total volume of 200 µl of complete media. Non-specific (NS) monoclonal

antibody to anti-Norwalk virus VP1 (Chemicon, Temecula, CA) was used at 5 µg/100µl as a

control antibody. After an additional incubation of 18 h, GFP positive cells were collected for

flow cytometry, and virus titers were measured as $TCID_{50}$/ml. For flow cytometry cells were

washed with PBS and trypsinized before being fixed in 4% paraformaldhyde and resuspended in

250µl of PBS for analysis as described below. The virus titration (50% tissue culture infectivity

dose, $TCID_{50}$) was performed as described below.

Treatments of MARC-145, PAMs, MDMs or CD14 Positive Monocytes with IL-10, TPA or LPS

MARC-145, PAMs, MDMs, or CD14 positive monocytes were treated with mock-EMEM, DMSO (0.1%), IL-10, TPA, or LPS to examine their effects on the expression of CD163 and PRRSV infectivity. In this study, LPS, TPA, or IL-10 was used at a concentration of 5 μg/ml, 50 μM, or 10 ng/ml, respectively. Trypan blue exclusion staining showed no cytotoxic effects at the concentrations used. Confluent MARC-145 cells were pretreated with mock-EMEM, DMSO, IL-10, TPA, or LPS for 2 h and then P129 was inoculated at a MOI of 1 for 1 h. The medium was then replaced with fresh medium containing the same treatment and incubated for additional 24, 48, or 72 h. After each time point, virus growth was enumerated by $TCID_{50}$ assay using MARC-145 cells.

For PAMs, cells were plated in 24-well plates at 5×10^5 cells/ml for 24 h before treatment with mock-EMEM, DMSO, IL-10, TPA, or LPS for 2 h. After the initial treatment, cells were infected with mock-medium, P129, or P129-GFP at a MOI of 1 and incubated in the presence of the same treatment for up to 72 h. The expression of CD163 and/or GFP was examined at 18 h after virus infection (P129-GFP) by flow cytometry. Virus (P129) growth was also measured at 12, 24, 48, and 72 h post-infection using $TCID_{50}$ assays in MARC-145 cells. For MDMs, CD14 positive cells (5×10^5 cells/ml in 24-well plates) were differentiated to macrophages in the presence of FBS and GM-CSF (10 ng/ml) for 3 days and then treated with each agent for 2 h before the inoculation with mock-medium, P129, or P129-GFP. After virus inoculation, cells were incubated in the presence of the same treatment for up to 72 h. Alternatively, freshly isolated CD14 positive monocytes (undifferentiated) (5×10^5 well^{-1} in 12-well plates) were

incubated in the presence of mock-EMEM, DMSO, TPA, IL- 10, or LPS in medium containing FBS and GM-CSF for 72 h. They were then inoculated with mock-medium, P129, or P129-GFP and further incubated for up to 72 h. The expression of CD163, GFP, or virus growth was measured at the same time points as PAMs.

Flow Cytometry Analysis for the Expression of CD163 or GFP

Flow cytometry analysis was used to compare the overall expression levels of CD163 and the infection levels of P129-GFP in PAMs and MDMs. All flow cytometry analysis was performed on a population of 10,000 cells using the FACS Calibur system (BD Bioscience, Franklin Lakes, NJ). Cells were prepared for flow cytometry analysis using the following protocol. Adherent cells were removed from the plate by incubation in 5mM EDTA/PBS for 2 h. Cells were washed with PBS and collected by centrifugation at 300×g for 10 min before being fixed with a 4% paraformaldehyde solution (Electron Microscopy Science, Hatfield, PA) while incubating on ice for 1 h. After the cells were washed in PBS, they were incubated with mouse anti-swine CD163 in PBS with 5% FBS on ice for 1 h. Cells were then washed in PBS and incubated in 5% FBS/PBS with a secondary R-Phycoerythrin conjugated (RPE) anti-mouse IgG1 antibody (Invitrogen) for 1 h. After washing the cells, they were analyzed for the presence of RPE on the flow cytometer. The same cells were also enumerated for the expression of GFP. Either single or dual color analysis was performed for RPE and/or GFP.

Data analysis of the flow cytometry data was performed using CellQuest[TM] (BD Bioscience). The appropriate gates were set using control cells which included cells without any treatment, PRRSV infected cells, and/or cells stained with only secondary conjugates. All quadrant gates

showing the expression of CD163 (RPE, y-axis) and GFP (PRRSV infection, x-axis) were set

using fixed cells only allowing for a maximum of a 1% overlap into any of the other three

quadrants. These gate settings were checked against cells stained with only secondary conjugates

and virus infected cells to ensure that they were appropriate for the experiment. Quadrant

statistics were obtained for the generation of the data sets in each figure. Positive populations

were identified as cells showing an increased florescence intensity level from the control cell

populations, and overall percentage levels of positive cells were calculated based on the quadrant

gates.

PRRSV Titration

$TCID_{50}$ assay was used for the PRRSV titration to examine the effects of the treatment on virus

replication in MARC-145, PAMs, CD14 positive monocytes, and MDMs. The titration was done

after virus infected cells were freeze-thawed twice to ensure complete cell lysis. After

centrifugation, the supernatant was assayed using the standard method on MARC-145 cells.

Statistical Analysis

All experiments were done with at least three independent experiments and statistical

significance ($p < 0.05$) was calculated with the Students' t-test.

Results

LLC-PK Cells Expressing CD163 Support the Replication of PRRSV

The full length CD163 cDNA (~3.6 kb, Fig. 2-1) was recovered from PAMs by RT-PCR, and

cloned into pIRES-Puro2. LLC-PK-CD163 cells that were selected in the presence of puromycin

(4 µg/ml) had similar growth kinetics as the parental cells (LLC-PK) with the majority of them

expressing CD163 (>80%) as confirmed by immunofluorescent assay (IFA) using an antibody against porcine CD163 (data not shown).

When parental LLC-PK cells were inoculated with either PRRSV strains P129 (or P129 strain carrying GFP gene [P129-GFP]) or SD01-08, there was no evidence of virus replication at 12, 24, or 48 h by IFA with SDOW17 (an anti-PRRSV nucleocapsid antibody conjugated with fluorescein isothiocyanate [FITC]) or GFP expression (Fig. 2-2). It has been demonstrated that the infectivity of P129-GFP is comparable to that of parental P129 (55). LLC-PK cells supported PRRSV replication when transfected with a P129 infectious cDNA, indicating that the lack of permissiveness was the result of the absence of a receptor on the cell surface. PRRSV replication was observed in LLC-PK-CD163 after inoculation with P129, P129-GFP or SD01-08 (Fig. 2). PRRSV infected cells were detected after 12 h with increasing numbers at 24 h with the appearance of fluorescent foci. By 48 h post-infection, the majority of cells were infected with PRRSV determined by IFA or observation of GPF expression. The course of infection correlated with the progression of CPE. However, neither fluorescence or CPE was observed in parental LLC-PK cells after infection with PRRSV strains (Fig. 2). These data confirm the observations of Calvert *et al.* and further support the notion that expression of CD163 can make cells permissive for PRRSV replication.

CD163 Expression Correlates with PRRSV Infectivity in Cells of Monocyte Origin

We utilized dual color flow cytometry to analyze PRRSV replication and CD163 expression in PAMs and MDMs during infection with PRRSV. For these experiments, surface CD163 expression was measured using RPE-labeled antibody, and virus replication in individual cells

was detected by the presence of GFP following infection with P129-GFP. Cells were infected at different times after placement into culture, and flow cytometry was performed 18 h later just prior to lysis of infected cells. For PAMs, a majority of cells expressed high levels of CD163 from days 0 to 3 (Fig. 2-3A). At day 0, over 60% of PAMs were CD163 positive. By days 2 and 3, the percentage of CD163 positive cells increased to more than 70%. The percentage of PAMs expressing GFP after the inoculation with P129-GFP was also high and ranged from 20 to 30% (Fig. 2-3B). After 48 h, the majority of virus infected PAMs were lysed (data not shown), indicating that most of cells were eventually infected with PRRSV. For CD14 positive monocytes, the expression level of CD163 was less than 10% at Day 0 (Fig. 2-3C). However, as cells were differentiated to MDMs, the population expressing CD163 steadily increased, reaching nearly 70% by day 3 (Fig. 2-3). The increased percentage of CD163 positive cells was not the result of CD163 negative cells dropping out, since cell numbers remained relatively constant over the culture period. A similar pattern was observed for the percentage of cells expressing GFP after infection (Fig. 2-3D). While there were few GFP expressing MDMs among cells at day 0, over 30% of cells were expressing GFP when incubated in culture longer than 2 days (Fig. 2-3D).

Anti-Porcine CD163 Antibody Reduced PRRSV Infection in PAMs

PAMs were incubated with various concentrations of anti-porcine CD163 antibody. At 18 h post-infection flow cytometry analysis showed that anti-porcine CD163 was capable of blocking virus infection in a dose dependent fashion. At the concentrations of 5µg/100µl or 3µg/100µl anti-porcine CD163 antibody reduced overall infection rate by 19.4% or 8%, respectively (Fig. 2-4A). However at the level of 1µg/100µl, the antibody showed little effect on PRRSV infection

(Fig. 2-4A and 2-B). Control monoclonal antibody (anti-Norwalk virus VP1) did not change the level of GFP expression. The $TCID_{50}$ results confirmed that GFP expression showing that there were greater than a 1.5 log difference at 5μg/100μl, and 1 log difference at 3μg/100μl anti-porcine antibody when compared to mock-treatment (Fig. 2-4B). These data suggest that the expression CD163 in PAM plays a significant role in determining the cells susceptibility to PRRSV infection.

Correlation of CD163 Expression and PRRSV Infectivity

Because the expression of CD163 can be modulated by various cytokines and reagents, we examined the effects of IL-10, TPA, or LPS on CD163 expression and PRRSV infection on MARC-145, PAMs or MDMs. For these experiments, mock-treated cells were incubated with DMSO, the solvent used to dissolve the TPA. There were no significant differences in CD163 expression (89%±2 vs. 90%±3) and GFP expression (34%±3 vs. 29%±11) when PAMs were infected with P129-GFP and treated with mock-medium or DMSO (0.1%). Therefore, we used DMSO-treatment as a control for comparison. The treatment of MARC-145 cells with TPA, IL-10, or LPS did not yield any significant differences in PRRSV replication as measured by virus yield (Fig. 2-5). However, after a 2 hr treatment with TPA, PAMs showed a reduction in the percentage of cells expressing CD163 (22%) and GFP (15%; Fig. 6A and Table 1).

The decreased percentage of dual positive cells in the TPA treated cultures corresponded with a significant decrease in virus yield, when measured at 12 and 24 h after infection ($p < 0.05$; Fig. 2-6B). The PAMs treated with IL-10 and LPS and infected with P129-GFP did not show significant changes in the overall expression of CD163 or GFP (Fig. 2-6A and Table 2-1). It was

observed that PAMs treated with LPS frequently yielded a reduced titer (about 0.7 log) at 12 h after virus infection, but was not significantly different over several experiments (Fig. 2-6B). The treatment of PAMs with IL-10 showed no significant changes in virus titers at any of the time points (Fig. 2-6A and Table 2-1). At 36 h post-infection, there was no significant difference in virus titers among the various treatments (Fig. 2-6B).

Modulation of CD163 on MDMs and PRRSV Infectivity

The effects of TPA, LPS or IL-10 on CD163 and PRRSV infectivity were examined in differentiated MDMs, or freshly isolated CD14 positive monocytes undergoing differentiation. Differentiated MDMs were prepared by incubating freshly isolated CD14 positive monocytes in the presence of 10 ng/ml GM-CSF for up to 4 days. Similar to PAMs, differentiated MDMs infected with P129-GFP after treatment with TPA demonstrated a significant ($p < 0.05$) reduction in the overall population expressing CD163 compared to mock(DMSO)-treated cells. This reduction correlated with the reduced number of GFP expressing cells (Fig. 2-7A and Table 2-1). Similar to PAMs, there was a subpopulation of cells that expressed relatively high levels of CD163, but were GFP negative. In addition, another subpopulation of cells expressed relatively low levels of CD163 on their surface, but were GFP positive. The cells expressing high levels of CD163 and low levels of GFP may represent the use of an alternative receptor, or perhaps the down regulation of surface CD163 expression following infection. Treatment of differentiated MDMs with LPS also reduced the percentages of CD163 and GFP expressing cells (Fig. 2-7A and Table 2-1). Differentiated MDMs treated with IL-10 and then infected with P129-GFP did not yield significant changes in the populations expressing CD163 or GFP (Fig. 2-7A and Table 2-1). Virus titers after each treatment and P129-GFP infection in the differentiated MDMs

correlated well with the expression of CD163 and GFP with the lowest virus yields obtained from cultures treated with TPA and LPS (Fig. 2-7B).

Experiments were also performed in freshly isolated CD14 positive monocytes incubated with GM-CSF in combination with DMSO, TPA, LPS, or IL-10, which resulted in drastic changes in the expression of CD163 and PRRSV infectivity. The treatment of freshly isolated CD14 positive monocytes with TPA or LPS resulted in the reduction of the number of cells expressing CD163 to less than 5% from approximately 50% of the control (Table 2-1). Interestingly, treatment of freshly isolated CD14 positive monocytes with IL-10 for 72 h significantly increased ($p < 0.05$) the numbers of cells expressing CD163 compared to the treatment with DMSO (Fig. 2-8A and Table 2-1). The titers of PRRSV after each treatment on freshly isolated CD14 positive monocytes also correlated with the overall levels of CD163 expressing cells (Fig. 2-8B and Table 2-1). The treatment of IL-10 significantly increased ($p < 0.05$) the PRRSV titers up to 1.5-fold at 12, 24, or 48 h after virus inoculation, the treatment with TPA or LPS yielded less than 1 log $TCID_{50}$ titers at all time points (Fig. 2-8B).

Discussion

PRRSV has a cellular tropism for cells of monocytic lineage, especially differentiated macrophages, such as porcine alveolar macrophages (21). Several putative receptors for PRRSV have been described, including heparin sulfate (10), sialoadhesin (9), and vimentin (24). Delputte *et al.* (10) identify heparin sulphate as a binding receptor on macrophages for both Type I and II PRRSV strains. However, it is not believed that heparin sulphate mediates the internalization of PRRSV(10). Porcine sialoadhesin has been identified as the molecule

responsible for the internalization of the PRRSV virion (9, 48). Monoclonal antibodies directed against sialoadhesin can prevent the internalizing the virus in cells (9). Kim *et al.* (24) reported that a monoclonal antibody (7G10) which binds vimentin has the ability to block PRRSV infection in MARC-145 cells, suggesting that it may be a possible receptor on MARC-145 cells (24). Recently, CD163 has been identified as a possible cellular receptor for PRRSV (4). Using a cDNA library created from total RNA isolated from PAMs, Calvert *et al.* (4) were able to identify CD163 as a receptor which allowed previously non-permissible cells to become susceptible to PRRSV infection with the expression of CD163. They demonstrated that this molecule alone could produce a fully susceptible cell line for both Type I and II PRRSV isolates (4). In this report, we confirmed CD163 as a receptor for PRRSV with the generation of a susceptible LLC-PK-CD163 cell line. This stable cell line expressing CD163 also fully supported the PRRSV strains of both Type I (SD01-08) and Type II (P129). The CD163 that was isolated in this study from total RNA of PAMs was the full length mRNA containing all 9 SRCR domains which was slightly different from a CD163 splice variant with 7 SRCR domains used by Calvert *et al.* (2007). Our study demonstrates that as a receptor for PRRSV, CD163 alone (either a full length or a variant) can bind and internalize the viruses for successful infection.

CD163, formerly known as M130 and RM3/1, has been identified as a hapten/hemaglobin scavenger receptor in the SRCR superfamily (38, 39, 53). There are 2 known porcine splice variants (susCD163v1 [Genbank: DQ067279] and susCD163v2 [Genbank: NM 213978) encoding for CD163 that have currently been identified in swine, while a number of variants have been identified in humans (4). The predominant mRNA form encodes an open reading frame of 3348 bp which consists of 9 SRCR domains, a transmembrane segment of 24 amino

acids, and a cytoplasmic tail comprised of 49 amino acids (18, 39). CD163 is currently thought

to be involved in anti-inflammatory responses and the removal of haptoglobin and hemoglobin

from serum (17). Interestingly, among macrophages from various tissues, the expression of

CD163 was highest on alveolar microphages and macrophages in the term placenta (17). This

may correlate with the major symptoms of PRRSV in animals: respiratory symptoms and

abortion.

Although CD163 is known to be expressed on monocyte lineages, not much is known regarding

its expression levels on PAMs and MDMs in the correlation with PRRSV infectivity. As

expected, a high number of PAMs that expressed CD163 were susceptible to PRRSV infection

regardless of incubation times in vitro (Fig. 2-3A and B). On the other hand, most of the freshly

isolated monocytes did not express CD163 and were not susceptible to PRRSV infection (Fig. 2-

3C and D). The expression of CD163 on MDMs was dependent on the differentiation levels and

correlated well with PRRSV susceptibility in our study (Fig. 2-3C and D). Blocking assays with

anti-porcine CD163 antibodies also suggested that CD163 may be the receptor on PAM that is

responsible for its susceptibility to PRRSV (Fig. 2-4A and B). These studies suggested that the

overall expression levels of CD163 on both PAMs and MDMs may determine the levels of

PRRSV replication and pathogenicity in vivo. It would be interesting to examine the expression

levels of CD163 on the macrophages from different tissues and the correlation with PRRSV

infection.

To further demonstrate evidence of the correlation between CD163 expression levels and

PRRSV infectivity in PAMs and MDMs, we modulated the expression of CD163 on the cells

and examined the effects on virus replication. It has been demonstrated that the expression of

CD163 on macrophages can be up-regulated by glucocorticoids and anti-inflammatory cytokines

such as IL-10, while down-regulated by pro-inflammatory mediators such as LPS, interferon

(IFN)-α, and tumor necrosis factor (TNF)-α (2). Therefore, we examined the effects of TPA, IL-

10, or LPS on CD163 expression and PRRSV infection in MARC-145 cells, PAMs, or MDMs.

We found that the treatment of in MARC-145 cells with TPA, IL-10, or LPS did not show

significant differences in PRRSV susceptibility, indicating these cells may be lacking the

pathway for CD163 modulation and that these agents did not affect PRRSV infectivity. It has

been previously demonstrated that the treatment of macrophages with protein kinase C activators

such as TPA or phorbol 12-myristate 13-acetate (PMA) causes a reduction in the surface

expression of CD163 (22). Furthermore, Duan *et al.* (11) reported that the treatment of

monocytes and macrophages with PMA reduced replication of PRRSV. Our results are

consistent with the data, demonstrating the treatment of PAMs or MDMs with TPA reduces the

population of cells expressing CD163 with a concurrent reduction of PRRSV susceptibility

(Figs. 2-5–7). Previously, Buechler *et al.* (2) showed that LPS down-regulated CD163 expression

on macrophages. In this study, while the treatment of PAMs with LPS did not cause a significant

reduction of CD163, the treatment of differentiated MDMs with LPS significantly reduced the

population expressing CD163 in correlation with the reduction of PRRSV infectivity (Fig. 2-7

and Table 2-1). The effects of TPA or LPS on CD163 expression and PRRSV infection were

more obvious when CD14 positive monocytes were treated during differentiation into MDMs

(Fig. 2-8). The treatment of these differentiating cells with TPA or LPS for 72 h yielded a drastic

decrease in the population of cells expressing CD163 (Fig. 2-8). The expression of GFP (after

P129-GFP infection) and PRRSV titers were nearly undetectable (Fig. 2-8). Our results suggest

that TPA or LPS may impair differentiation of monocytes and/or the expression of CD163.

Cytokines play an important role in the regulation of the overall immune response, both

enhancement and inhibition. IL-10, an anti-inflammatory cytokine, has been shown to inhibit the

activation of a number of cells involved in the immune response including T cells, monocytes,

and macrophages. IL-10 can inhibit the expression of various cytokines by monocytes and

macrophages including IL-1α, IL-1β, IL-6, IL-12, IL-18, GM-CSF, M-CSF, TNF, leukemia

inhibitory factor (LIF) and platelet-activating factor (PAF) (7, 14, 19, 50, 51). The primary role

of IL-10 appears to be the termination of the inflammatory response (43). Several different

viruses including Epstein-Barr, equine herpes virus type 2, poxvirus Orf virus, and human

cytomegalovirus encode an IL-10 homolog suggesting an important role in their replication

cycles (15, 25, 30, 42, 49). In this study, we found that the incubation of freshly isolatedCD14

positive cells with IL-10 during differentiation increased the susceptibility to PRRSV infection in

correlation with the up-regulation of CD163 on the cell surface (Fig. 2-7). This suggests a

significant role for IL-10 in CD163 and PRRSV susceptibility during the differentiation of

macrophages in vivo. However, the treatment of PAMs and differentiated MDMs with IL-10 did

not significantly change overall expression levels of CD163 and PRRSV infectivity. This may be

due to the fact that most of these cells were already expressing CD163. Interestingly, it has been

shown that the endocytosis of CD163 upon stimulation with hemoglobin or haptaglobin has the

ability to increase the overall production of IL-10 (34). It is possible that the internalization of

PRRSV via CD163 in the target cells may induce the expression of IL-10, and in turn induce the

70

expression of CD163 on neighboring undifferentiated monocytes and increase overall PRRSV susceptibility. We are currently studying this possibility using PAMs and MDMs.

Proinflammatory cytokines such as IL-1β, IL-8 and TNF-α have been shown to be upregulated after treatment of monocytes or macrophages with LPS (32, 41). LPS plays an important role in PRRSV infections probably due to coinfections with gram negative bacteria. Coinfection of PRRSV and other microbes such as *Mycoplasma hyponeumoniae* (45), *Streptococcus suis* (46), and type 2 porcine circovirus (44) is commonly observed in the field. It has been shown that coinfection with PRRSV and each of these pathogens can cause an increase of the overall gross pathology (36, 45-47). Pigs with a dual infection of PRRSV and *M. hyponeumoniae* exhibited an increased duration and severity of PRRSV infection (45). This may be due to the increased recruitment of monocytes caused by the pro-inflammatory cytokines in response to bacterial infections followed by anti-inflammatory cytokines such as IL-10 as the immune system tries to counter the inflammatory response. The modulation of the expression of CD163 on PAMs or MDMs by cytokines or other agents in vitro suggest that PRRSV susceptibility may be affected by the presence of such molecules due to inflammation or co-infections with various bacteria and viruses in the target tissue. In summary, our data demonstrated that CD163 alone can confer PRRSV replication in a non-permissible pig cell line, and the expression of CD163 on monocytes/macrophages in different microenvironments in vivo may determine the replication of PRRSV and the virus pathogenicity. Understanding the replication of PRRSV with the regulation of CD163 on the monocytes/macrophages in vitro and in vivo should provide important information regarding virus pathogenicity and preventive measures against the devastating disease.

Acknowledgements

Table 2-1. The Cell Population (%) Expressing CD163 or GFP After Each Treatment and Infection with P129-GFP for 18 h in PAMs, CD14 Positive Undifferentiated Monocytes or Differentiated MDMs.

[a] The population expressing CD163 or GFP by the treatment was significantly different from that of mock(DMSO)-treatment ($p < 0.05$).

	DMSO	TPA	LPS	IL-10
PAMs				
CD163	90 ± 3	77 ± 6^a	88 ± 3	91 ± 5
GFP	29 ± 11	14 ± 7^a	25 ± 5	28 ± 9
MDMs				
CD163	85 ± 6	30 ± 15^a	67 ± 11^a	90 ± 3
GFP	34 ± 6	15 ± 13^a	21 ± 13^a	33 ± 9
CD14 Positive				
CD163	50 ± 3	1 ± 0.4^a	1 ± 1^a	78 ± 2^a
GFP	15 ± 7	0.5 ± 0.3^a	0.7 ± 0.5^a	20 ± 8^a

Figure 2-1. mRNA and Structural Organization of CD163.

CD163 is approximately 120 kDa in size and amember of the scavenger receptor cysteine-rich

(SRCR) superfamily. It is composed of 3 primary domains including 9 SRCRdomains, a

transmembrane region, and a short cytoplasmic tail. (A) The mRNA organization of CD163. (B)

The structural organizations of CD163 with full 9 domains (upper panel) or a splice variant with

7 domains (lower panel).
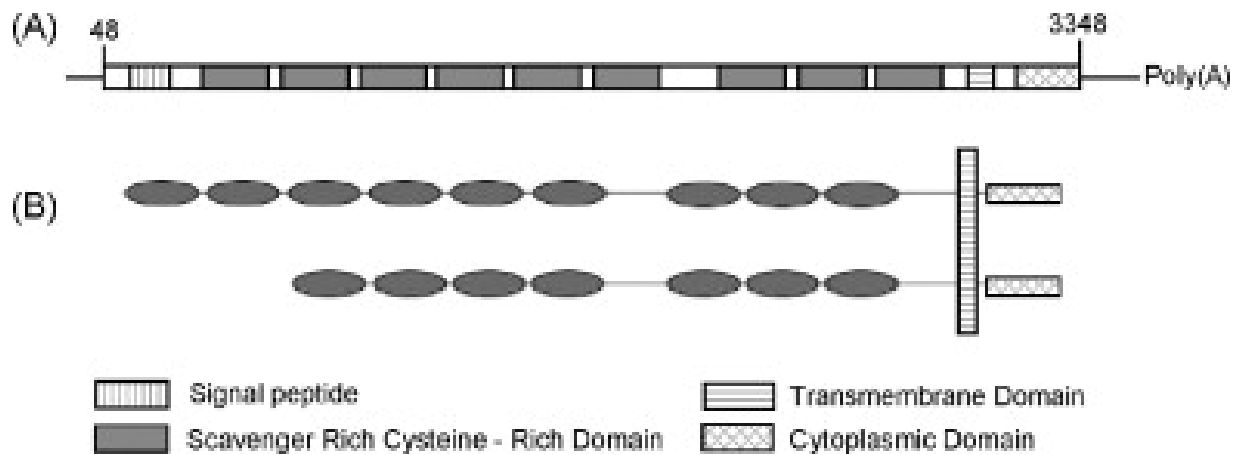
Figure 2-2. PRRSV Replication in Parental LLC-PK or LLC-PK-CD163 cells.

Confluent parental LLC-PK or LLC-PK-CD163 cells were infected with P129 or P129-GFP for

up to 48h, and virus replication was detected by IFA (A) or GFP expression (B). (A) LLC-PK or

LLC-PK CD163 cells infected with P129 for 24 h. Fixed cells were incubated with SDOW17

and observed under a fluorescent microscope. (B) LLC-PK or LLC-PK-CD163 cells infected

with P129-GFP for 24 or 48 h, and observed under a fluorescent microscope (upper panel) or a

light microscope (lower panel).
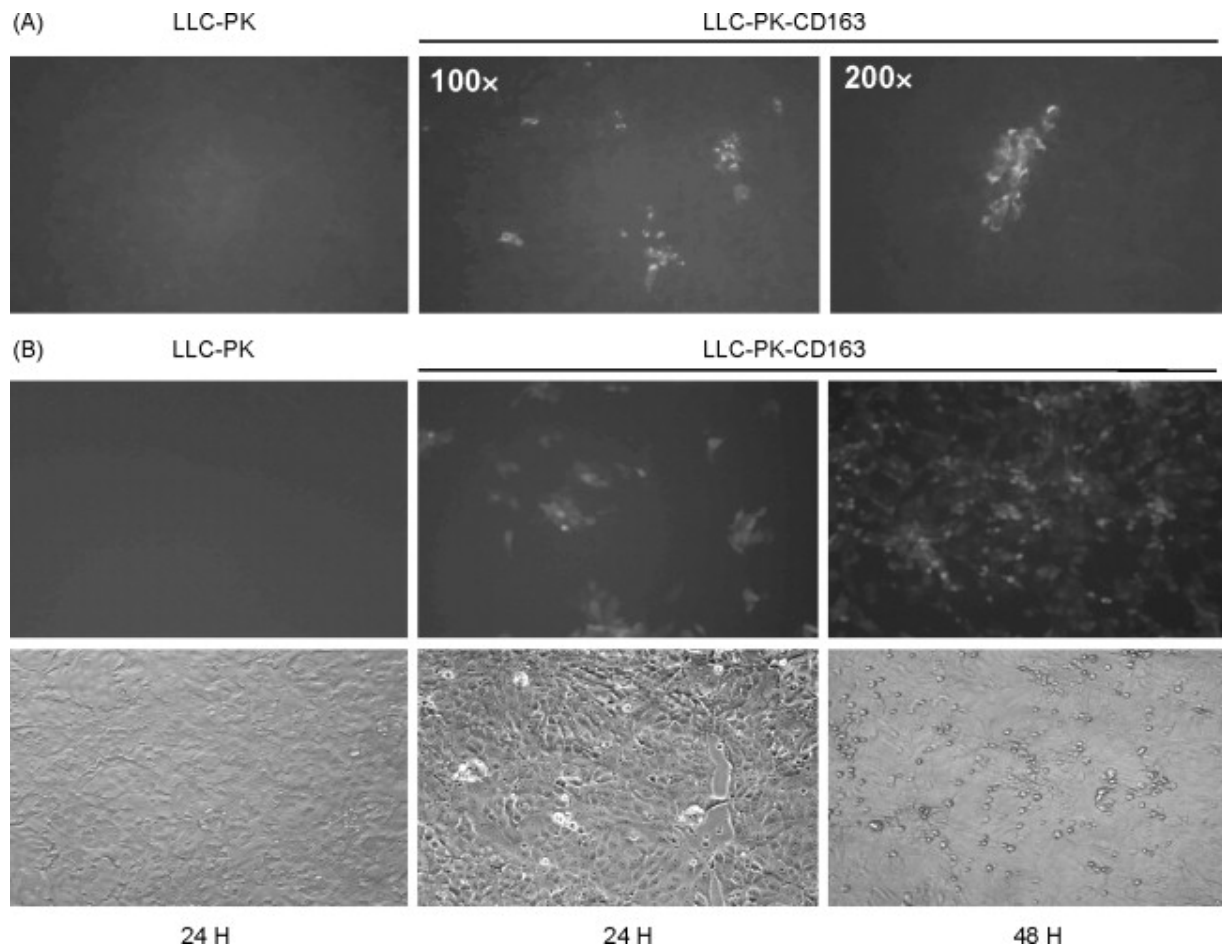
Figure 2-3. The Expression of CD163 and PRRSV Infectivity in PAMs and Differentiating CD14 Positive Monocytes.

PAMs and freshly isolated CD14 positive monocytes were placed in culture for 0, 1, 2, and 3 days, infected with PRRSV (P129-GFP) for 18 h and enumerated for the population expressing CD163 or GFP by flowcytometry. (A–D) The population expressing CD163 (A and C) or GFP (B and D) on 0, 1, 2, and 3-day-old PAMs (A and B) or differentiating CD14 positive monocytes (C and D). Bars represent standard error of at least three independent experiments in all figures.

Figure 2-4. The Effects of Anti-CD163 Antibody on the Infection of PAMs with P129-GFP.
Cellswere incubated with 0, 1, 3, or 5µg anti-porcine CD163 antibody per 100µl complete
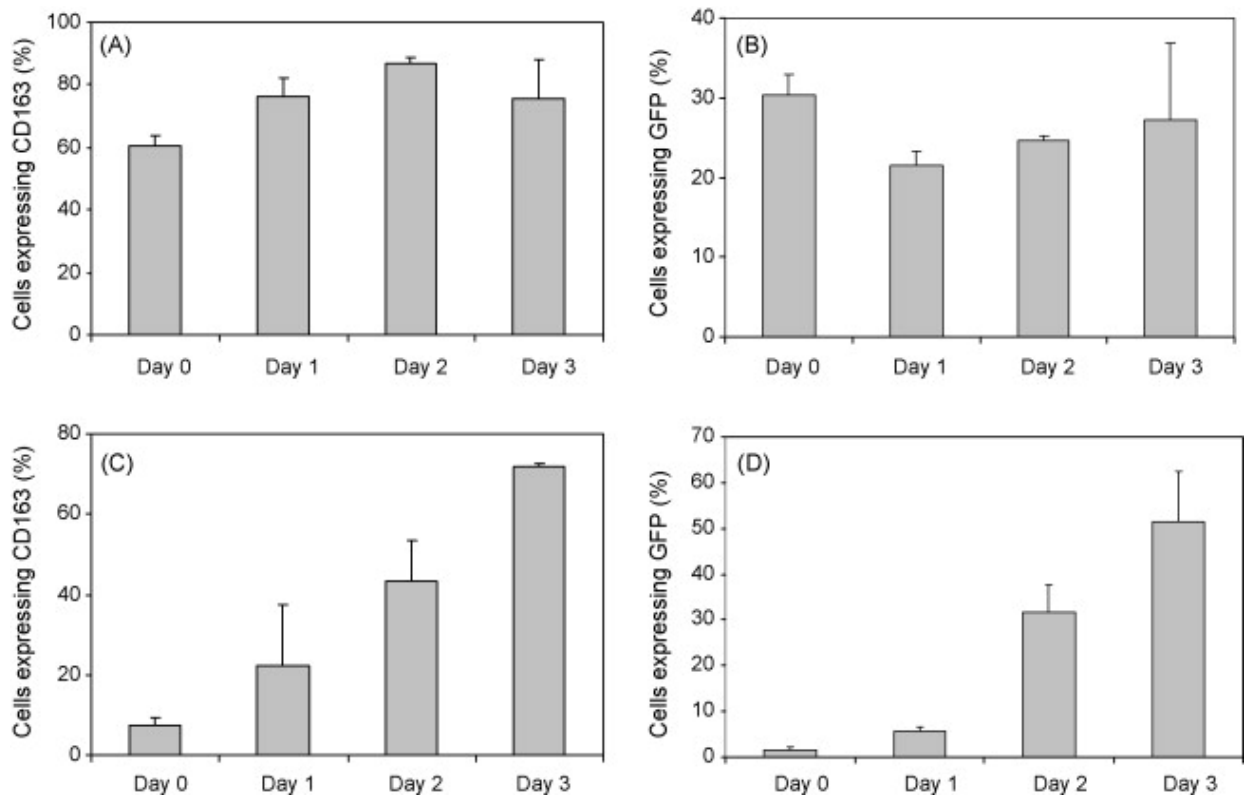
medium for 1 h before infection with P129-GFP. Non-specific anti-Norwalk virus IgG was used

as a control antibody. At 18 h post-infection the percentage of GFP-expressing cells was

determined by flow cytometry. Cells were fixed with 4% paraformaldehyde before overall GFP

expression was measured. Grey line represents untreated cells, each black line represents a

treatment group. The left shift in each treatment group represents the overall decrease in GFP

expression levels (4A). $TCID_{50}$ levels were also measured 18 h post-infection (4B). Error bars

represent the standard error of 3 separate experiments. Asterisk (*) indicates that the PRRSV

titer by the treatment was significantly reduced from that of mock-treatment ($p < 0.05$).

Figure 2-5. The Effects of TPA, LPS, or IL-10 on PRRSV Infection in MARC-145.

Confluent MARC-145 cells were pretreated with DMSO (mock-control), TPA (50µM), LPS

(5µg/ml), and IL-10 (10 ng/ml) for 2 h before they were inoculated with P129 at a MOI of 1. The

virus infected cells were further incubated in the presence of the same treatment for 24, 48, 72 or

96 h, and $TCID_{50}$ virus titerswere determined using MARC-145 cells.
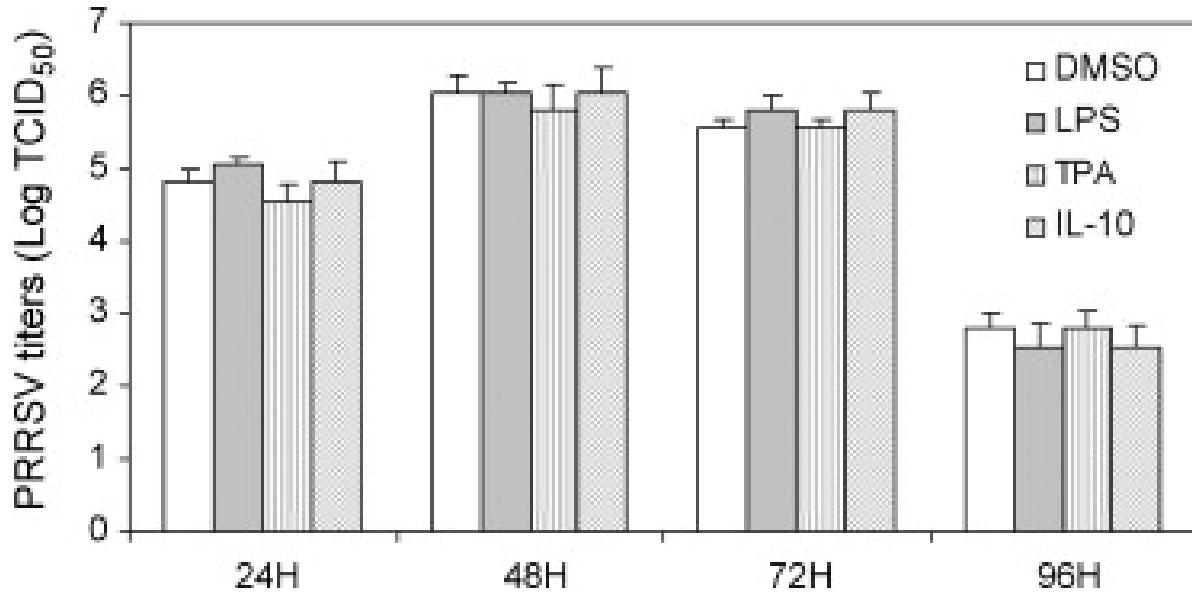
Figure 2-6. The Effects of DMSO, TPA, LPS, or IL-10 on PRRSV Replication in PAMs.
One-day-old PAMs (5×10^5 cells/well in 12-well plates) were treated with DMSO (mock-control), TPA (50μM), LPS (5μg/ml), and IL-10 (10 ng/ml) for 2 h, and then they were inoculated with either mock-medium, P129 or P129-GFP at a MOI of 1 and incubated in the presence of the same treatment up to 36 h, and examined for the expression of CD163 or GFP and for virus replication. (A) The expression of CD163 (RPE [R-Phycoerythrin], y-axis) or GFP (x-axis) examined by flow cytometry analysis after the pretreated cells were infected with P129-GFP for 18 h. This flow cytometry data is froma representative animal and a part of Table1. (B) PRRSV titers after the pretreated PAMs were infected with P129 for 12, 24 and 36 h. Asterisk (*) indicates that the PRRSV titer by the treatment was significantly reduced from that of mock-treatment ($p < 0.05$).
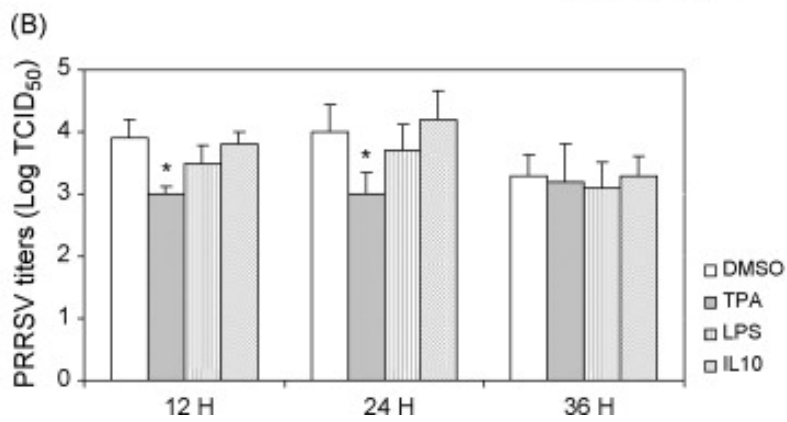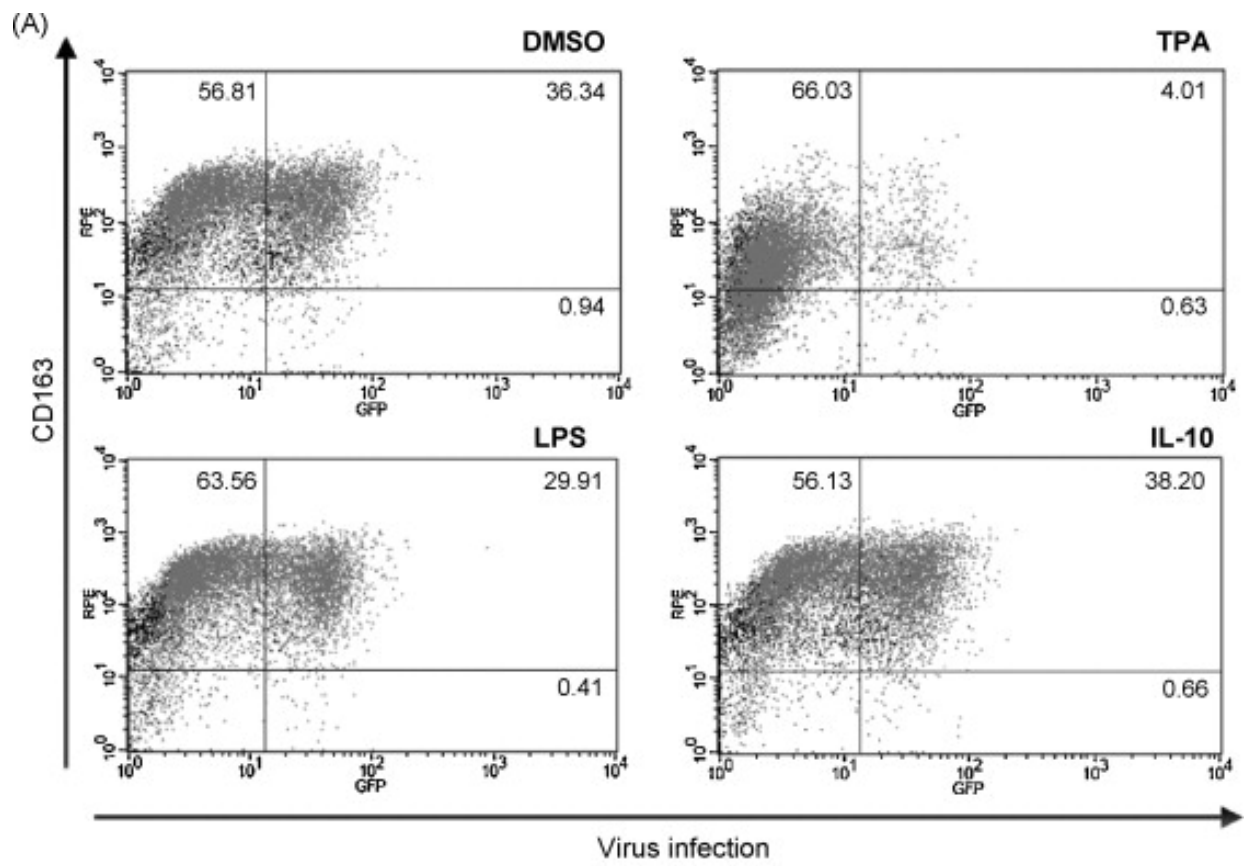
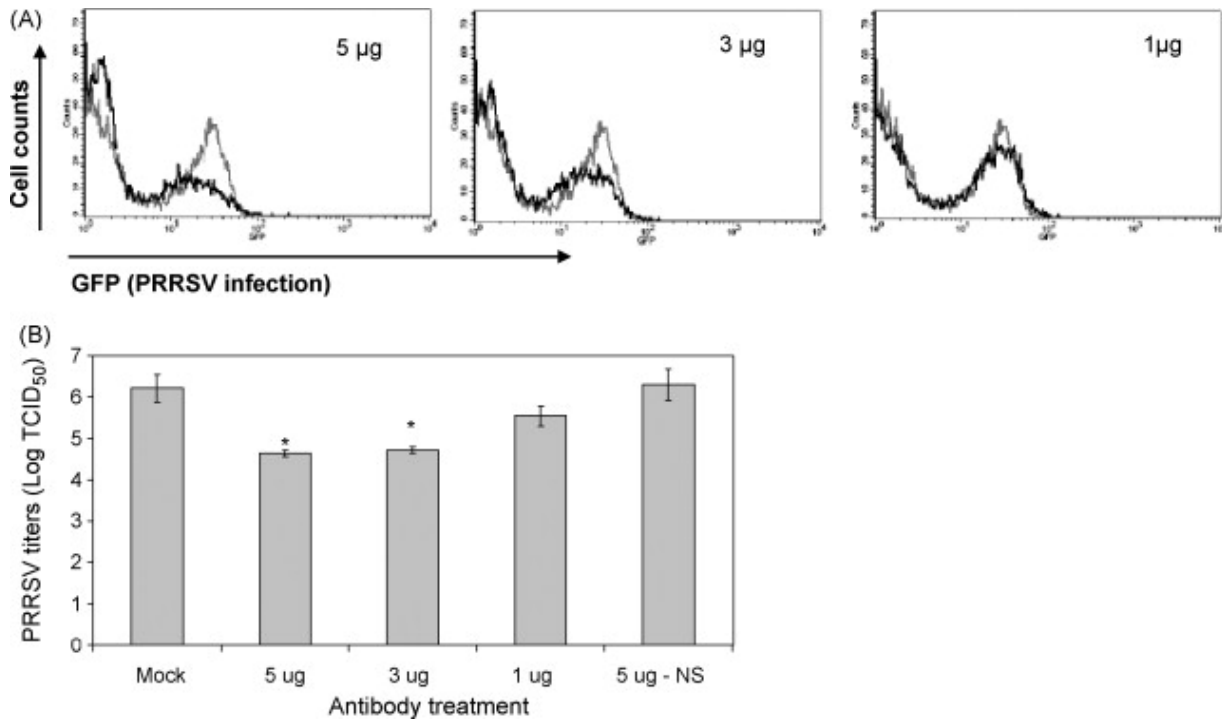**Figure 2-7. The Effects of DMSO, TPA, LPS, or IL-10 on PRRSV Replication in Differentiated MDMs.**

Isolated CD14 positive monocytes were incubated in the presence of GM-CSF (10 ng/ml) and 10% FBS for at least 3 days for the differentiation to macrophages. The differentiated MDMs were treated with DMSO(mock-control), TPA (50μM), LPS (5μg/ml), and IL-10 (10 ng/ml) for 2 h, and then they were inoculated with either mock-medium, P129 or P129-GFP at a MOI of 1. Virus infected cells were further incubated for in the presence of the same treatment up to 24 h. (A) The expression of CD163 (RPE [R Phycoerythrin], y-axis) or GFP (x-axis) examined by flow cytometry analysis after the pre-treated cells were infected with P129-GFP for 18 h. This flow cytometry data is from a representative animal and a part of Table 1. (B) PRRSV titers after the pretreated MDMs were infected with P129 for 12 and 24 h. Asterisk (*) indicates that the PRRSV titer by the treatment was significantly reduced from that of mock-treatment ($p < 0.05$).

(A)

DMSO
32.32   16.68
              13.25

TPA
5.98   0.73
            3.21

LPS
41.12   4.15
            4.99

IL-10
36.80   17.74
            9.08

CD163

Virus infection

(B)

PRRSV titers (Log TCID$_{50}$)

12 H   24 H

□ DMSO
■ TPA
▦ IL10
▨ LPS

82
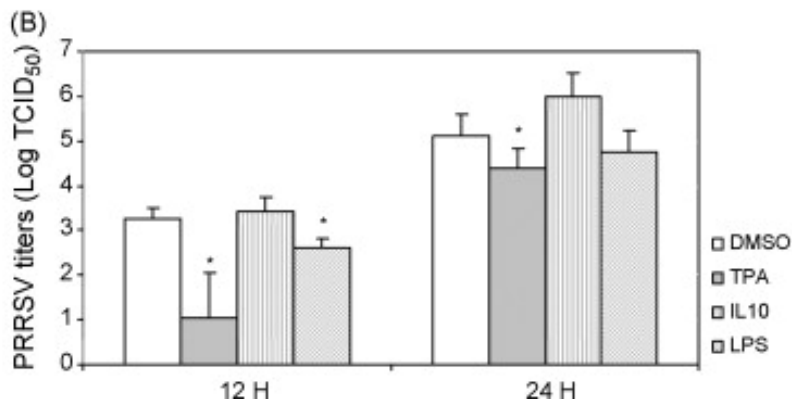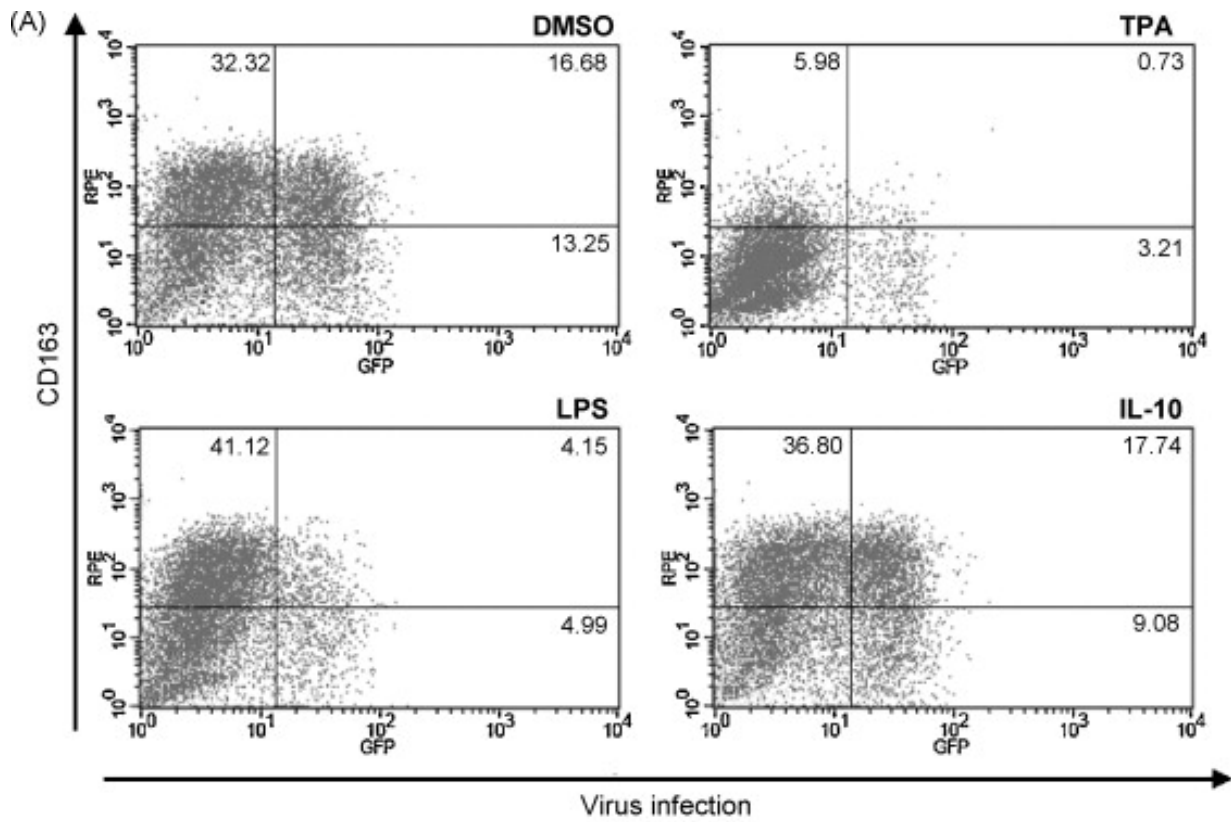
Figure 2-8. The Effects of DMSO, TPA, LPS, and IL-10 on PRRSV Replication in CD14 Positive Monocytes.

Freshly isolated CD14 positive monocytes were treated with DMSO (mock-control), TPA (50μM), LPS (5μg/ml), and IL-10 (10 ng/ml) for 72 h, and they were inoculated with either mock-medium, P129 or P129-GFP at a MOI of 1. Virus infected cells were further incubated in the presence of the same treatment for up to 72 h. (A) The population expressing CD163 after the pretreated cells were infected with P129-GFP for 18 h. (B) PRRSV titers after the pretreated CD14 positive monocytes were infected with P129 for 24, 48 and 72 h. Asterisk (*) indicates that the population expressing CD163 (A) or the PRRSV titer (B) by the treatment was significantly different from that of mock treatment ($p < 0.05$).
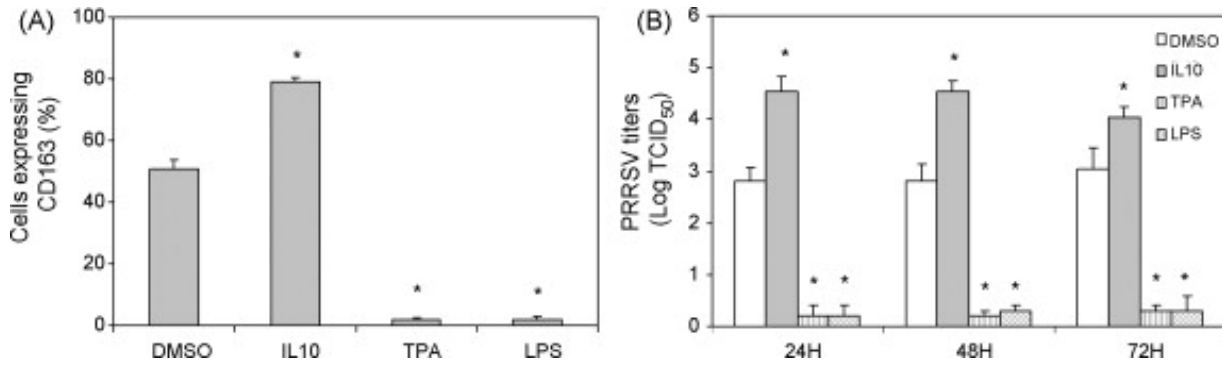
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CHAPTER 3 - Bile Acids Promote HCV Replication Through the EGFR/ERK Pathway in Replicon-Harboring Cells

Abstract

Hepatitis C virus (HCV) undergoes chronic infections in hepatocytes, which frequently results in liver cirrhosis and cancer. There are currently over 170 million people persistently infected with HCV worldwide, and spontaneous clearance of the virus is rare. Recently, we demonstrated that bile acids promoted the replication of HCV and compromised the anti-HCV effects of interferon alpha (IFN-α) in replicon-harboring cells. To explore a potential mechanism for our observation, we studied the effects of bile acids on the epidermal growth factor receptor (EGFR) and the extracellular signal regulated kinase (ERK) pathway in association with HCV replication in replicon-harboring cells containing either genotype 1a or 1b. First, we found that various bile acids (chenodeoxycholic acid, glycochenodeoxycholic acid, deoxycholic acid and ursodeoxycholic acid) significantly increased the expression of a reporter protein through the activator protein-1 (AP-1) and serum responding element (SRE), and this was correlated with the activation of EGFR and an extended S-phase during cell cycle progression in the presence of bile acids. Second, the inhibitors of EGFR (AG1478) or mitogen-activated protein kinase kinase 1/2 (U0126) significantly mitigated the bile acid-mediated promotion of HCV replication in the cells. Finally, when AG1478 or U0126 were added to the treatment of bile acids and IFN-α, they were able to restore the anti-HCV effects of IFN-α in replicon-harboring cells. Our data suggest that the addition of an EGFR or ERK inhibitor to the current IFN-α based regimen may improve overall treatment efficacy by blocking the bile acid-mediated promotion of HCV replication.

Introduction

Hepatitis C virus (HCV) is a single-stranded, positive sense RNA virus with a genome length of approximately 10 kb (10, 21, 22, 24). HCV can persist as a chronic infection in hepatocytes, and the spontaneous clearance of an HCV infection is rare (24, 27). Furthermore, chronic HCV infections frequently lead to liver cirrhosis and cancer (2, 31). The current standard treatment for chronic HCV infection is a combination therapy consisting of pegylated interferon alpha (IFN)-α and ribavirin (13, 29). Both IFN-α and ribavirin have been shown to be cost effective and demonstrate significant antiviral effects against a HCV (6, 43, 46). The combination therapy is very effective against HCV genotype 2 and 3 viruses resulting in sustained virological response rate of around 80%. However, the effectiveness of the combination treatment is low (40-50%) in patients infected with HCV genotype 1 (27). The lack of an effective treatment for HCV genotype 1 serves as a motivation to gain further knowledge of the HCV replication process and its relationship with the host cells.

While several HCV strains have recently been isolated that have been successfully tissue culture adapted, the majority of HCV remains fastidious in cell culture systems (24, 42, 44, 45, 47). As a solution to the lack of tissue culture adapted viruses a cell based virus replication system, replicon-harboring cells were established and have provided an excellent tool for the study of virus replication and the development of antivirals (4, 25, 40). In our previous study, we demonstrated that bile acids promoted HCV replication and compromised the anti-HCV action of interferon (IFN)-α using replicon-haboring cells containing HCV genotype 1b (GS4.1 cells) (8). Our study suggested that bile acids may be a reason for low efficacy of IFN based treatment in a large percentage of patients with chronic HCV infections (8). Various bile acids including

chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA) all promoted HCV replication and interfered with IFN-α action in the replicon-harboring cells (8). Scholtes *et al.* (38) also demonstrated that bile acids enhanced the replication of genotype 1, but not genotype 2 HCV.

Bile acids have been shown to promote the proliferation of various cells including hepatocytes via several pathways including the epidermal growth factor receptor (EGFR) (14, 16-19, 34). It has been suggested that the highest levels of HCV RNA and proteins are observed while the replicon-harboring cells were undergoing active division (1, 32), suggesting that HCV replication may be associated with the cell cycle progress. Therefore, we hypothesized that bile acids promote HCV replication in replicon-harboring cells by modulating cell cycle progress via the EGFR/extracellular signal regulated kinase (ERK) pathway in replicon-harboring cells. In this report, we found that bile acids led to the activation of the promoter activities of activator protein 1 (AP-1) and serum responding element (SRE) in replicon-harboring cells. We also demonstrated that bile acids modulated the cell cycle progression by extending S stage, which was associated with the phosphorylation of EGFR and enhanced levels of both HCV RNA and protein in the cells. In addition, the inhibitors of either EGFR (AG1478) or the mitogen-activated protein kinase kinase (MEK) 1/2 (U0126) blocked bile acid-mediated promotion of HCV replication in the cells. Finally, we found that the interference of IFN-α action by bile acids was mitigated in the presence of the EGFR or ERK inhibitor. Our data suggest that the addition of an EGFR or ERK inhibitor to the current IFN-α based regimen may improve overall treatment efficacy by blocking the bile acid-mediated promotion of HCV replication.

Materials and Methods

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Cell Lines and Reagents.

Huh-7, GS4.1 (replicon-harboring cells with the HCV 1b genotype, provided by Dr. C. Seeger at the Fox Chase Cancer Center, Philadelphia, PA), and 1A7 (replicon-harboring cells with the HCV 1a genotype, provided by Dr. S. Lemon at the University of Texas Medical Branch at Galveston, Galveston, TX) were maintained in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal bovine serum. Both GS4.1 and 1A7 cells were maintained in the presence of G418 (Cellgro, Manassas, VA) at 0.5 μg/ml. Antibodies against HCV NS5b, β-actin, phosphor-EFGR or EGFR were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bile acids, chenodeoxycholic acid (CDCA), glycol-chenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA), were obtained from Sigma-Aldrich (St. Louis, MO). GCDCA was resuspended in deionized-distilled water, while all unconjugated bile acids (CDCA, UDCA, and DCA) were resuspended in dimethyl sulfoxide (DMSO). IFN-α (recombinant IFN-αA/D human), AG1478 and U0126 were also obtained from Sigma-Aldrich.

The AP-1 and SRE Promoter Assay.

Replicon-harboring cells (GS4.1) were transfected with 2 μg of either pAP1-TA-Luc or pSRE-TA-Luc (Clonetech, Palo Alto, CA), and 2 μg of pRL-CMV (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 4 hr of transfection, cells were incubated in the fresh media containing CDCA (100 μM), GCDCA (200 μM), DCA (100 μM), UDCA (200 μM), or a combination of CDCA (100 μM) and U0126 (20 μM). Cells were further incubated for 24 hr before being lysed and subjected to luciferase assay using a Dual Glo luciferase assay

kit (Promega). The level of firefly luciferase expression was normalized against the expression level of the renilla luciferase encoded for by pRL-CMV.

Cell Cycle Analysis.

Semi-confluent GS4.1 or 1A7 cells were synchronized using aphidicolin (Sigma-Aldrich) at a concentration of 5 μM for 24 hr before being stimulated with CDCA (100 μM), GCDCA (200 μM), or CDCA (100 μM) + U0126 (20 μM) for 0, 6, 12, and 24 hr. For cell cycle analysis, cell monolayers were lysed with Vindelov's propidium iodide (PI) solution (35) and cell nuclei were strained through a cell strainer cap on 5 ml round bottom tubes (BD Biosciences, San Jose, CA). Nuclei were analyzed on a Beckman Dickenson flow cytometer and the cell division stages were analyzed using ModFit LT (VSH Software, Topsham, ME). Dual-staining examining cell cycle and the expression of HCV protein was performed on cells with or without the treatment of bile acids using the BD Cytofix/Cytoperm kit (BD Biosciences).   Briefly, after each treatment, cells were fixed and permeabilized in 200 μl of BD Fixation/Permeabilization solution with a 1:100 dilution of a mouse monoclonal NS5b antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. Cells were washed 3 times in BD Perm/Wash Buffer and incubated with a FITC-labelled goat-anti-mouse secondary antibody (Caltag [Invitrogen, Carlsbad, CA]) at a dilution of 1:100 in Perm/Wash Buffer for 2 hr. Following incubation, cells were washed 3 times in BD Perm/Wash buffer and transferred to either a PI solution or PBS and allowed to sit for 1 hr before analysis. Fifty thousand cells were analyzed on the FACSCalibur (Beckman Dickenson, Franklin Lakes, NJ) and NS5b levels were analyzed using the CellQuest (Beckman Dickenson). The specific percentage of cells at a particular stage in the cell cycle was determined using ModFit LT.

Treatment of Replicon-Harboring Cells with Bile Acids, Inhibitors of EGFR (AG1478) or MEK1/2 (U0126), and IFN-α

First, we examined the cytotoxic effects of each bile acid and/or the inhibitor on GS4.1 or 1A7 cells using a CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Medison, WI) to obtain the concentration of each agent with minimum cell toxicity. To examine the role of EGFR and the ERK pathway in bile acid-mediated promotion of HCV replication and/or interference with the anti-HCV action of IFN-α, AG1478 and U0126 were added in conjunction with various bile acid concentrations. Both GS4.1 and 1A7 cells were used in this study. First, the treatments were focused on the ability of EGFR or ERK inhibitors in blocking bile acid-mediated enhancement of the HCV replication. The treatments included AG1478 (15 or 30 μM), U0126 (10 or 20 μM), CDCA (100 μM), and CDCA in combination with AG1478 or U0126, using the concentrations listed above (Table 1). Cells were incubated with the various treatments for 24 hr before HCV RNA and protein levels were measured using real-time quantitative reverse transcription-PCR (qRT-PCR) and Western blot analysis as described in section 2.5. Second, the treatments were designed to examine the ability of the EGFR or ERK inhibitor in restoring the anti-HCV effects of IFN-α in the presence of bile acids. The treatments included AG1478 (15 or 30 μM), U0126 (10 or 20 μM), IFN-α (5 U/ml), CDCA (100 μM), CDCA + AG1478 or U0126, IFN-α + U0126 or AG1478, IFN-α + CDCA, and CDCA + IFN-α + AG1478 or U0126 (Table 1). The cells were incubated with the various treatments for 24 hr before HCV RNA and protein levels were measured using qRT-PCR and Western blot analysis, respectively.

Detection of HCV RNA and NS5b Protein.

The HCV RNA and NS5b protein levels were measured 24 hr after semi-confluent GS4.1 or 1A7 cells were treated with various bile acids, IFN-α and/or AG1478, and/or U0126 (Table 1) by qRT-PCR or western blot analysis, respectively. Total RNA was isolated from GS4.1 or 1A7 cells in 6-well plates following treatment using the RNeasy Mini Kit (Qiagen, Valencia, CA) and qRT-PCR was performed using a One-Step Superscript III qRT-PCR kit (Invitrogen) with primers and probes previously described (8). Western blot analysis was performed on cells lysed with lithium dodecyl sulfate (LDS) sample buffer containing 2% β-mercaptoethanol. NS5b protein was detected using a mouse monoclonal anti-NS5B antibody at a 1:1000 dilution in 2% non-fat dry milk (NFDM) followed by a secondary HRP conjugated goat-anti-mouse (Pierce Biosciences, Rockford, IL) at 1:1000. Protein was visualized using a chemiluminescent substrate (Pierce Biosciences). β-actin levels were measured by qRT-PCR and Western blot analysis for the normalization of RNA and protein levels of each sample, respectively. The expression of NS5B in cells with or without bile acid treatment was also examined by flow cytometry analysis as described above.

Detection of Phosphor-EGFR and EGFR.

The activation of EGFR was detected using Western blot analysis with phosphor-EFGR and EGFR antibodies. Briefly, semiconfluent GS4.1 cells were incubated with CDCA (100 μM), GCDCA (200 μM), or UDCA (200 μM) for 30 min before lysis with LDS sample buffer containing 2% β-mercaptoethanol. Western blot analysis was performed using an anti-phosphor-EGFR or EGFR antibody at 1:1000 dilution in 2% NFDM and a secondary anti-mouse HRP

conjugated detection antibody at 1:500. Proteins were visualized using a chemiluminescent

substrate as described above.

Statistical Analysis

Each experiment was repeated a minimum of three times. Statistical analysis was performed

using GraphPad Prism 5.0 data analysis software.

Results

Bile Acids Increased HCV Replication in 1A7 cells.

In a previous study, we demonstrated that bile acids promoted the replication of HCV RNA and

proteins in GS4.1 cells containing a genotype 1b replicon. In this report, we found that various

bile acids also increased the levels of HCV RNA and protein in 1A7 cells containing a genotype

1a replicon. 1A7 cells were treated with mock-medium, CDCA, GCDCA, or UDCA, and HCV

RNA or NS5b was measured by real-time qRT-PCR, or Western blot analysis or flow cytometry

analysis, respectively. After incubation with 100 μM or 20 μM of CDCA for 24 hr, HCV RNA

levels in 1A7 cells were significantly increased ($p<0.05$) to 226% ± 14 or 163% ± 19 compared

to mock treatment (100%). The levels of HCV RNA were further increased to 312% ± 12 and

237% ± 13 compared to mock-treatment after a 48 hr incubation. The incubation of cells with

200 μM GCDCA or 100 μM UDCA also showed an increase in HCV RNA levels to 140% ± 9 or

185% ± 12 (24 hr) and 166% ± 31 or 206% ± 17 (48 hr) ($p<0.05$), respectively. The protein

levels of HCV NS5b correlated with HCV RNA levels after the incubation with CDCA 100 μM

(Figure 3-1B-Lane 2), with a significant enhancement in protein level compared to mock treated

cells (Figure 3-1B–Lane 1). Enhanced NS5b protein levels were observed in all other treatments

including CDCA 20 µM (Lane 3), GCDCA 200 µM (Lane 4), and GCDCA 100 µM (Lane 5). The flow cytometry analysis also confirmed the enhanced expression of NS5b by the treatment with bile acids: after treatment with 100 µM of CDCA for 24 hr, cells expressing NS5b had increased compared to mock treated cells (47% vs 33%) (Figure 3-1C).

Enhancement of Luciferase Activity Under AP1 or SRE Promoter After Bile Acid Treatment in GS4.1 Cells

Luciferase activity under the control of the AP-1 or SRE promoter was significantly increased (greater than a 1.8 fold, $p<0.05$) by the treatment with CDCA (100 µM), DCA (100 µM), GCDCA (200 µM), or UDCA (200 µM) for 24 hr compared to mock-medium (negative) treatment (Figure 3-2A). CDCA was most efficient among tested bile acids in increasing luciferase activity under AP-1 or SRE, which was consistent in promoting HCV replication in cells (Figure 1A) (8). The treatment of the cells with U0126 blocked the bile acid-mediated enhancement of luciferase activity (Figure 3-2B), which further suggests that bile acids induce the activation of the signalling pathways of AP-1 or SRE in the cells.

Bile Acids Induce a Prolonged S Phase Period in Replicon-Harboring Cells

Since AP-1 and SRE are known to play a role in modulation of the cell cycle, we analyzed cell cycle progression of GS4.1 and 1A7 cells after treatment with mock-medium, CDCA, GCDCA, and U0126 + CDCA for up to 24 hr (Figure 3-3 and Table 3-2). For 1A7 cells, cell cycle progression with G1, S, or G2 stages showed no significant difference between mock, CDCA, or

GCDCA at 6 hr post treatment (Table 3-2). However, 1A7 cells in S phase were significantly increased ($p<0.05$) at 12 hr post treatment with CDCA or GCDCA when compared to mock treated cells: 86% (CDCA) and 81% (GCDCA) vs 67% (Mock) (Table 2). At 24 hr, 1A7 cells in S phase were also significantly increased ($p<0.05$) by treatment with CDCA compared to mock-treatment (40% vs 14%) (Table 3-2). For GS4.1 cells, cell cycle progression was similar to 1A7 cells with or without bile acids for 6 or 12 hr with the treatment with CDCA or GCDCA also increased the percentage of cells in S phase at 12 hr (Figure 3-3A). By dual-staining examining NS5b positive cells and cell cycle progression with the treatment of mock-medium or CDCA for 24 hr, both cells at S-stage and cells expressing NS5b increased by CDCA (Figure 3-3B). In the presence of U0126 (20 µM), cell cycle progression with CDCA was delayed at 6 hr (Figure 3-3A).

Bile Acid-Mediated Promotion of HCV Replication is Correlated with EGFR Pathway.

We determined bile acids induced the activation of EGFR in replicon-harboring cells. When GS4.1 cells were incubated with medium containing mock-medium, CDCA, UDCA, or GCDCA for 30 min, the phosphorylation of EGFR was only evident in cells with bile acids (Figure 3-4). To examine the role of an EGFR inhibitor in the bile acid-mediated enhancement of HCV replication, semi-confluent GS4.1 or 1A7 cells were incubated with mock-medium, CDCA, AG1478 or CDCA + AG1478 for 24 hr, and HCV replication was measured. The treatment with AG1478 alone did not change HCV RNA levels in the cells, but when AG1478 was co-treated with CDCA (100 µM), it significantly reduced ($p<0.05$) CDCA-mediated enhancement of HCV RNA levels at the concentrations above 15 µM. The treatment with CDCA increased HCV RNA

levels to over 200% compared with the mock-treatment in both GS4.1 and 1A7 cells. However, the HCV RNA levels were significantly ($p<0.05$) decreased to 151% ± 4 or 135% ± 5 in GS4.1 and 1A7 cells respectivly in the presence of 30 μM of AG1478 (plus CDCA) (Figure 3-5A). Like AG1478, U0126 mitigated bile acid-mediated promotion of HCV replication in GS4.1 or 1A7 cells, while U1026 (10 or 20 μM) alone did not significantly alter the HCV RNA level (Figure 3-5B). While the treatment with CDCA increased HCV RNA levels to over 200%, in the presence of U0126 (plus CDCA), the HCV RNA levels were significantly ($p<0.05$) decreased to 126% ± 11 or 158% ± 7 (GS4.1) and 109% ± 14 or 175% ± 5 (1A7) at 20 or 10 μM, respectively (Figure 3-5B). The levels of HCV NS5b were well correlated with HCV RNA levels after treatment with AG1478 and/or bile acids in replicon-harboring cells (Figure 3-5C).

The Inhibitor of EGFR or ERK Restores the Anti-HCV Action of IFN-α in the Presence of Bile Acids.

Because bile acids compromise the anti-HCV action of IFN-α, we examined if AG1478 or U0126 could restore IFN-α action in the presence of bile acids. GS4.1 or 1A7 cells were treated with IFN-α alone (5 U/ml), double treatment of IFN-α and CDCA (100 μM) or triple treatment of IFN-α, CDCA and AG1478 (30 μM) or U0126 (20 μM). The levels of HCV RNA were measured after 24 hr of treatments. In GS4.1 cells the treatment with IFN-α reduced the HCV RNA levels to 39% ± 3 of those with mock-treatment; but in the presence of CDCA, it was reduced to only 97% ± 8 ($p<0.05$ compared to IFN-α treatment). With the triple treatment with A1478 or U0126, the HCV RNA levels were 64% ± 8 or 50% ± 10 ($p<0.05$ compared to double treatment of IFN-α and CDCA) of those treated with mock-treatment (Figure 3-6). We found similar results with 1A7 cells. While the treatment with IFN-α (5 U/ml) reduced the HCV RNA

levels to 51% ± 4 of those with mock-treatment, with the double treatment of IFN-α and CDCA, HCV RNA levels were 102% ± 7. The triple treatment of IFN-α, CDCA and A1478 or U0126 resulted in the HCV RNA levels to 53% ± 16 or 60% ± 5 of those receiving mock-treatment (Figure 6). These results suggest the inhibitor of EGFR or ERK restores the anti-HCV action of IFN-α in the presence of bile acids.

Discussion of Results

While several putative receptors of HCV, including CD81 (9, 12, 28, 33) and human scavenger receptor class B1 (15, 26, 37, 41), are distributed throughout the body, HCV mainly replicates within hepatocytes in the liver. This suggests there are hepatocyte-specific cofactors that determine the liver tropism of HCV. Previously we demonstrated that bile acids, both conjugated and unconjugated, can play an important role in the up-regulation of HCV replication in replicon-harboring cells. In the body, most bile acids present as conjugated forms with taurin or glycin, and they are collected and stored in the gall bladder at concentrations reaching as high as 320 mM before released into the upper small intestine (duodenum) (20). Most of bile acids returned to the liver through the enterohepatic circulation via the portal vein where concentrations of bile acids can reach up to 80 μM as it passes into the liver (23). In this report, we demonstrated that the activation of the EGFR/ERK pathway may play an important role in the bile acid-mediated enhancement of HCV replication. In 2004, Carloni *et al.* (7) demonstrated that CD81, a putative receptor for HCV, has the ability to activate the ERK pathway. In addition, Brazzoli *et al.* (5) demonstrated that activation of the ERK pathway by CD81 was necessary for specific cellular events required for the replication HCV. When the authors blocked the ERK signalling cascade using the MEK 1/2 inhibitor (U0126), viral replication was virtually abolished. Previous studies have shown that bile acids are important in

the normal regeneration of the liver (14, 16), and they activate the ERK pathway in primary rat hepatocytes with the EGFR receptor (34). The ERK pathway stimulates both AP-1 and the SRE through a series of intermediate proteins, including the ternary complex factor (TCF) subfamily of proteins (11). In accordance with literature we demonstrated that bile acids activate the ERK pathway, and found that bile acids led to the enhanced expression of luciferase under both the AP-1 and SRE promoters in replicon-harboring cells (Figure 3-2A). In addition, the total luciferase activity was significantly reduced when the replicon-harboring cells were treated with 100 μM CDCA in combination with 20 μM U0126 (Figure 3-2B). This suggests that signalling though the ERK pathway is induced by bile acids and leads to the activation of both AP-1 and SRE. The AP-1 complex is comprised of components from the jun oncogene (JUN), v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), activating transcription factor (ATF), and v-maf musculoaponeorotic fibrosarcoma oncogene homolog (MAF) protein families (39). FOS and JUN are the main proteins in mammalian systems which form AP-1 complexes and regulate gene expression. AP-1 has the ability to be both oncogenic and anti-oncogenic through the regulation of different sets of genes, including those involved in cell proliferation (3, 39).

Because both AP-1 and SRE lead to cell proliferation, we examined the cell cycle progression of replicon-harboring cells in the presence of bile acids using flow cytometry analysis. It has been previously reported that cell cycle progression was important in HCV replication, with the highest levels of HCV RNA were observed while cells are actively undergoing division in replicon-harboring cells (42). We observed that upon stimulation with various bile acids, 1A7 and GS4.1 cells entered into extended S phase (Figure 3-3, Table 3-2), which correlated with the enhanced expression levels of HCV RNA and proteins (Figure 3-1A-C). The cell cycle

progression of GCDCA treated cells had a shorter S phase hold than its CDCA counterpart. This

shorter S phase hole correlates with the lower overall levels of HCV RNA and protein seen after

stimulation with bile acids. The extended S phase that is induced by bile acids may be caused by

the continuous stimulation of the AP-1 and SRE promoters mediated by the constitutive

activation of the ERK pathway in the presence of bile acids. The activation of the ERK pathway

upon binding of HCV to CD81, and the continuous stimulation by bile acids suggest that the

ERK pathway may play an important role in the overall life-cycle of HCV. Furthermore, both

the HCV core and NS5b proteins have been demonstrated to induce a high percentage of cells to

hold in S phase when overexpressed in a non-neoplastic human hepatocyte cell line (30, 36),

suggesting that active replication of HCV may enhance cells in S phase, consequently further

increase virus replication. However, it is not clear what exact mechanism(s) are involved in

enhanced virus replication in S staged cells. It is possible that proteins expressed in cells in S

stage may act as co-factors for virus replicase, and we will examine this possibility in the future.

When GS4.1 cells were treated with either CDCA, GCDCA, or UDCA, the phosphorylation of

EGFR was seen in the presence of each bile acid after 30 min (Figure 3-4), suggesting an

important role of EGFR in AP-1 and SRE activation and the cell cycle progress in the presence

of bile acids. A 30 min time point was chosen because previously published data shows high

levels of EGFR phosphorylation after bile acid stimulation in rat hepatocytes (Rao et al. 2002).

To study the effect of the activation of the EGFR/ERK pathway on bile acid-mediated

enhancement of HCV replication and the interference with the anti-HCV effects of IFN-α, we

used AG1478 and U0126. While AG1478 alone had little effect on HCV replication, it

significantly reduced CDCA-mediated promotion of HCV replication at concentrations above 15

μM in both GS4.1 and 1A7 cells (Figure 3-5A). Similar to AG1478, U0126 had little effect on

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the HCV replication alone, but when combined with CDCA it significantly mitigated the bile acid-mediated enhancement of HCV (Figure 3-5B). These data suggest that the ERK pathway is playing an important role in the bile acid-mediated enhancement of HCV RNA in replicon-harboring cells. Since we previously demonstrated that bile acids compromise the anti-HCV action of IFN-α, we hypothesized that AG1478 and/or U0126 can restore the IFN-α action in replicon-harboring cells in the presence of bile acids by reducing the overall bile acid-mediated enhancement of the HCV replicon. The anti-HCV effects of IFN-α were significantly improved when GS4.1 and 1A7 cells were treated with the combination of CDCA and IFN-α with either U0126 or AG1478 (Figure 3-6). These data suggest that the addition of an EGFR or ERK inhibitor, such as AG1478 or U0126 to the current IFN-α based treatment may improve overall efficacy by blocking the bile acid-mediated increase in HCV replication. In summary, we demonstrated that bile acids induce EGFR/ERK pathway in genotype 1a and 1b replicon-harboring cells, which was associated with increased levels of HCV replication. Disruption of this signalling by either AG1478 or U0126 can significantly reduce the bile acid-mediated promotion of HCV and restore the anti-HCV effects of IFN-α in the cells in both genotypes.

ACKNOWLEDGEMENTS

Table 3-1. List of Treatment of Replicon-Harboring Cells with Bile Acids, Inhibitors of EGFR or ERK, and IFN Alone or Together in this Study.

Treatment	Compounds (concentrations)
Mono-treatment	CDCA (100 μM, 20 μM); GCDCA (200 μM, 100 μM); DCA (100 μM); UDCA (100 μM); AG1478 (30 μM, 15 μM); U0126 (20 μM, 10 μM); IFN-α (5 U/ml)
Dual-treatment	CDCA (100 μM) + IFN-α (5 U/ml); CDCA (20 μM) + IFN-α (5 U/ml); CDCA (100 μM) + AG1478 (30 μM); CDCA (100 μM) + AG1478(15 μM); CDCA (100 μM) + U0126 (20 μM); CDCA (100 μM) + U0126 (10 μM); AG1478 (30 μM) + IFN-α (5 U/ml); U0126 (20 μM) + IFN-α (5 U/ml);
Triple-treatment	AG1478 (30 μM) + CDCA (100 μM) + IFN-α (5 U/ml); U0126 (20 μM) + CDCA (100 μM) + IFN-α (5 U/ml);

Table 3-2. Cell Cycle Analysis of 1A7 Cells Incubated with Mock-Medium, CDCA (100 µM) or GDCDA (200 µM) for 6, 12, or 24 hr.

Treatment	Percentage (%) of cells[*]					
	0 hr			6 hr		
	G1	S	G2	G1	S	G2
Mock	75±3	25±3	0	25±16	75±15	1±1
CDCA	-	-	-	41±28	59±27	1±1
GCDCA	-	-	-	24±16	75±16	1±1
	12 hr			24 hr		
	G1	S	G2	G1	S	G2
Mock	10±1	67±10	23±10	67±2	13±3	20±2
CDCA	9±2	86±6**	6±4	26±4	40±4**	38±3
GCDCA	8±1	81±1**	11±0	61±4	14±4	25±1

Figure 3-1. Enhancement of HCV Replication After Bile Acid Treatment in 1A7 Cells.

Semi-confluent cells were treated with mock-medium, CDCA, GCDCA, or UDCA for 24 or 48 hr. HCV RNA (A) or NS5b (B) was measured by real-time qRT-PCR or Western blot analysis, respectively. A. qRT-PCR levels after treatment with mock-medium, CDCA 100 μM (CDCA100), CDCA 20 μM (CDCA20), GCDCA 200 μM (GCDCA200), GCDCA 100 μM (GCDCA100) and UDCA 200 μM (UDCA200) for 24 or 48 hr. Asterisk (*) indicates that the RNA levels by the treatment were significantly increased compared to those by control (mock-medium) treatment ($p<0.05$). B. Western blot analysis detecting NS5b after the treatment for 24 hr. Upper panel, lane 1: mock-medium; lane 2: CDCA (100 μM); lane 3: CDCA (20 μM); lane 4: GCDCA (200 μM); lane 5: GCDCA (100 μM). Lower panel, as a loading control, Western blot analysis of β-actin was performed with the same samples. C) Flow cytometry analysis of NS5b levels in 1A7 cells with the treatment of mock-medium or CDCA (100 μM) for 24 hr. Staining control was prepared the same procedure without the incubation with the NS5b antibody.
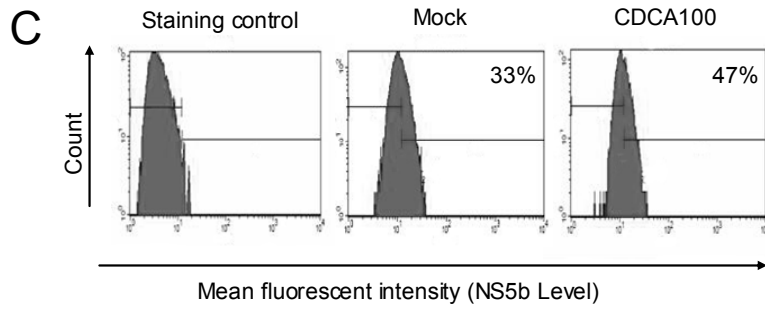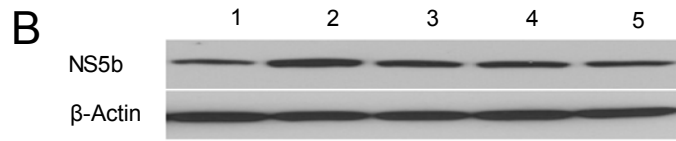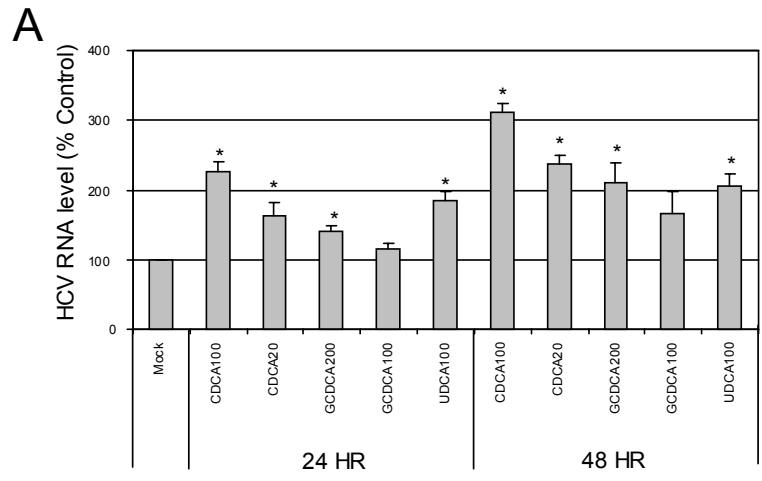
A

B

1 2 3 4 5

NS5b

β-Actin

C

Staining control   Mock   CDCA100

33%   47%

Count

Mean fluorescent intensity (NS5b Level)

Figure 3-2. Enhancement of Luciferase Activity Under AP1 or SRE Promoter Control After Bile Acid Treatment in GS4.1 Cells.

The luciferase based plasmid under AP-1 or SRE promoter control was transfected into semiconfluent GS4.1 cells. The cells were treated with CDCA 100μM (CDCA100), DCA 100μM (DCA200), GCDCA 200μM (GCDCA200), UDCA 200μM (UDCA200), or CDCA100 + U0126 20μM (U20) 4 hr after transfection and incubated for an additional 24 hr. Asterisk (*) indicates that the luciferase activities by the treatment were significantly increased compared to those by the control treatment ($p < 0.05$). A). Treatment of various bile acids in the luciferase activity under the control of signalling pathways, AP-1 or SRE. B). The effects of U0126 on CDCA-mediated induction of luciferase expression under AP-1 or SRE. Asterisk (*) indicates that the luciferase activities by co-treatment with CDCA and U0126 were significantly reduced compared to those by the treatment with CDCA alone ($p < 0.05$).
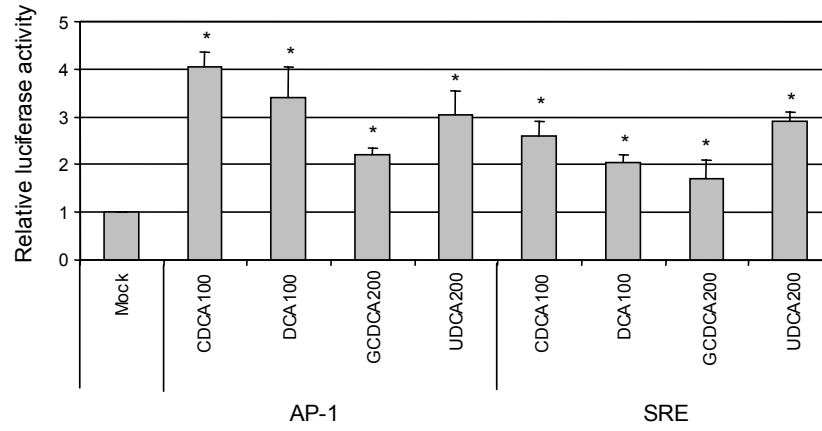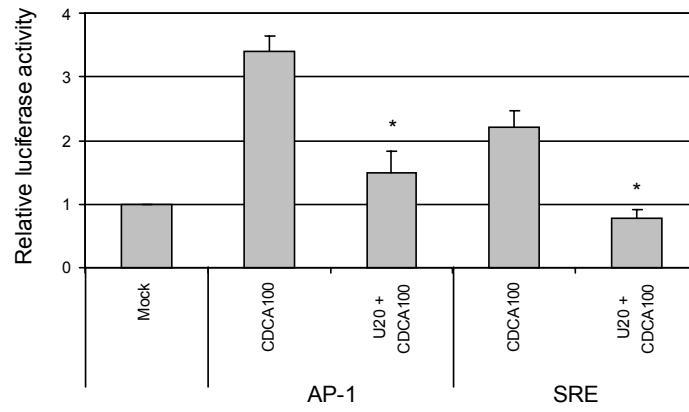
A

B

Figure 3-3 Cell Cycle Analysis of GS4.1 Cells After Treatment with Mock, CDCA, GCDCA, and U0126 + CDCA.

Semi-confluent GS4.1 cells were synchronized with aphidicolin at 5 μM for 24 hr before the treatment. Cells were treated with mock-medium, CDCA 100 μM (CDCA100), GCDCA 200 μM (GCDCA200), or CDCA100 + U0126 20 μM (U20) for 6 or 12 hr before being analyzed for cell cycle progression. A. Cells treated with CDCA 100 μM or GCDCA200 μM for up to 12 hr. B) Dual-staining examining cell cycle and the expression of HCV protein (NS5b). Cells were treated with mock-medium or CDCA 100 μM (CDCA100) for 24 hr.

A

Mock | CDCA 100 uM | GCDCA 200 uM

0 h
G1: 77%
S: 23%

6 h
G1: 9%
S: 90%
G2: 1%

G1: 13%
S: 86%
G2: 1%

G1: 8%
S: 91%
G2: 1%

12 h
G1: 10%
S: 57%
G2: 33%

G1: 11%
S: 80%
G2: 9%

G1: 9%
S: 80%
G2: 11%

CDCA + U0126, 6 h
G1: 87%
S: 7%
G2: 6%

CDCA + U0126, 12 h
G1: 11%
S: 86%
G2: 3%

Cell Count

FL2-A

B

G1: 21%
G2: 53%
S: 25%

14%

Mock

G1: 29%
G2: 39%
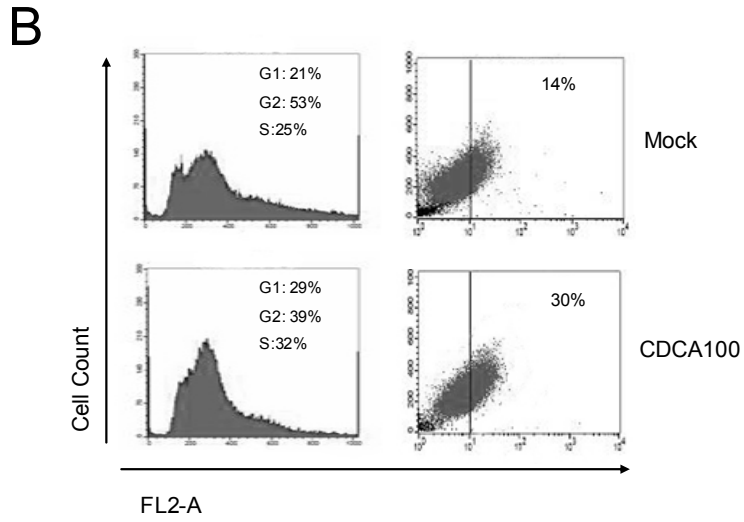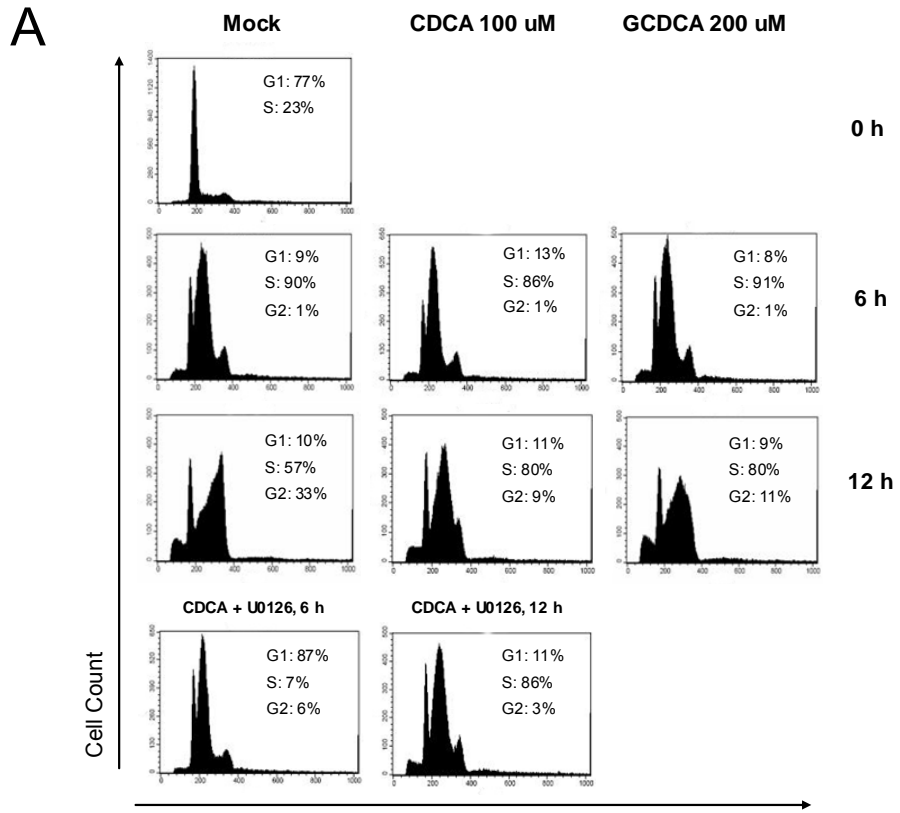S: 32%

30%

CDCA100

Cell Count

FL2-A

Figure 3-4. The Activation of EGFR by the Treatment with Bile Acids in GS4.1 cells.

Confluent GS4.1 cells were treated with mock-medium, CDCA, GCDCA, or UDCA for 30 min,

and then cell lysates were prepared and Western blot analysis detecting phosphor-EGFR or

EGFR. Lane 1: parental Huh7 cells with mock-treatment; lane 2: mock-treatment; lane 3:
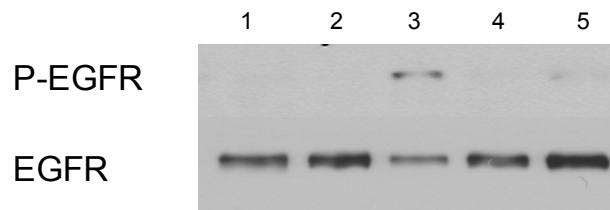
CDCA 100 μM; lane 4: GCDCA 200 μM; lane 5: UDCA 200 μM.

Figure 3-5. Inhibitors of EGFR or ERK in Bile Acid-Mediated Promotion of HCV Replication.

Semi-confluent GS4.1 or 1A7 cells were incubated with mock-medium, CDCA, AG1478 or CDCA + AG1478 for 24 hr, and HCV replication was measured by detecting HCV RNA (A and B) or protein (C). A. Real time qRT-PCR analysis of HCV RNA levels after the treatment with CDCA 100 μM (CDCA100), AG1478 30 μM (AG30), CDCA100 + AG30, or CDCA100 + AG1478 15 μM (AG15). B. Real time qRT-PCR analysis of HCV RNA levels after the treatment with CDCA100, U0126 20 μM (U20), CDCA100 + U20, or CDCA100 + U0126 10 μM (U10). Asterisk (*) indicates that the RNA levels by double treatment with CDCA and AG1478 or U0126 were significantly reduced compared to those by the treatment with CDCA alone ($p<0.05$). C. Western blot analysis of HCV protein NS5B in GS4.1 cells after treatment with CDCA, AG1478 or CDCA + AG1478. Lane 1: Mock, Lane 2: CDCA 100 μM, Lane 3: AG1478 30 μM + CDCA 100 μM, Lane 4: AG1478 5 μM + CDCA 100 μM, and Lane 5: AG1478 30 μM. As a loading control, Western blot analysis of β-actin was performed with the same samples.
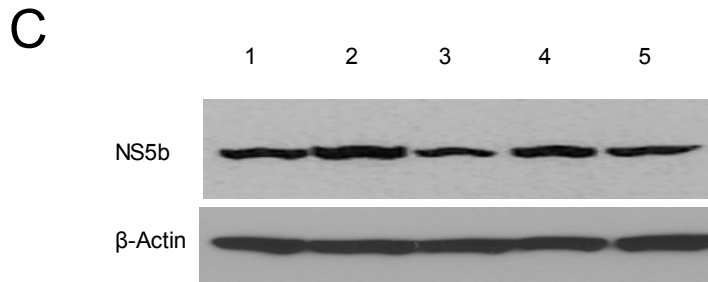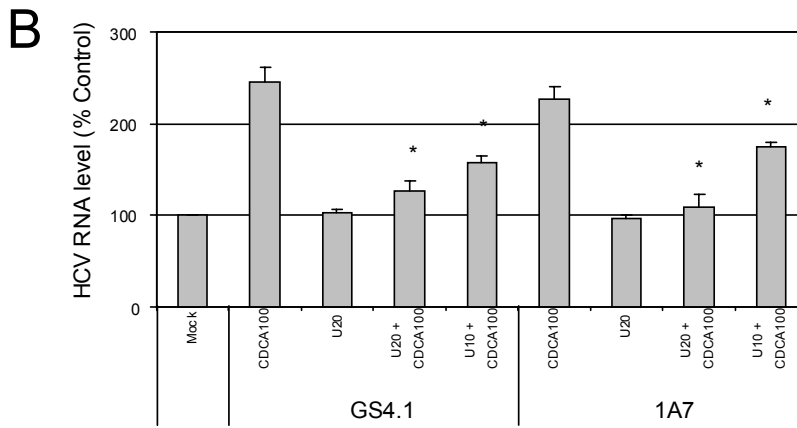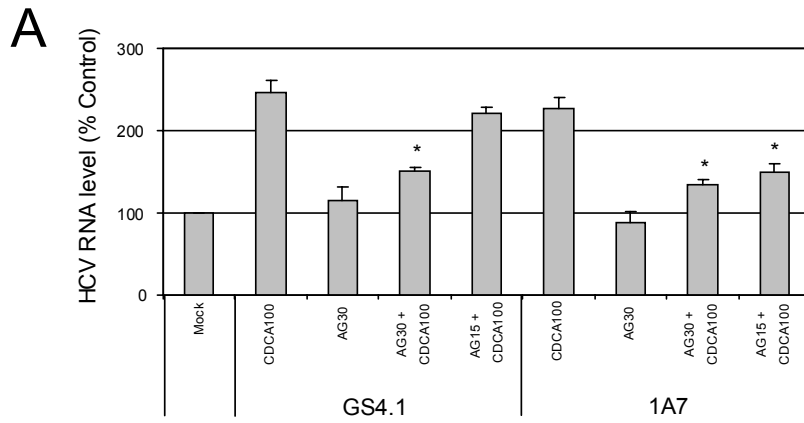
Figure 3-6. Inhibitors of EGFR or ERK in Anti-HCV Action of IFN in the Presence of CDCA.

HCV RNA levels were measured after 24 hr treatments with IFN 5 U/ml (IFN5), CDCA 100 μM (CDCA100) + IFN5, or CDCA100 + IFN5 + (AG1478 30 μM [AG30] or U0126 20 μM [U20]). Asterisk (*) indicates that the RNA levels by IFN 5 U/ml treatment were significantly reduced compared to those by mock-treatment ($p<0.05$). Double asterisk (**) indicates that the RNA levels by triple treatment were significantly reduced compared to those by double treatments of IFN5 + CDCA100 ($p<0.05$).
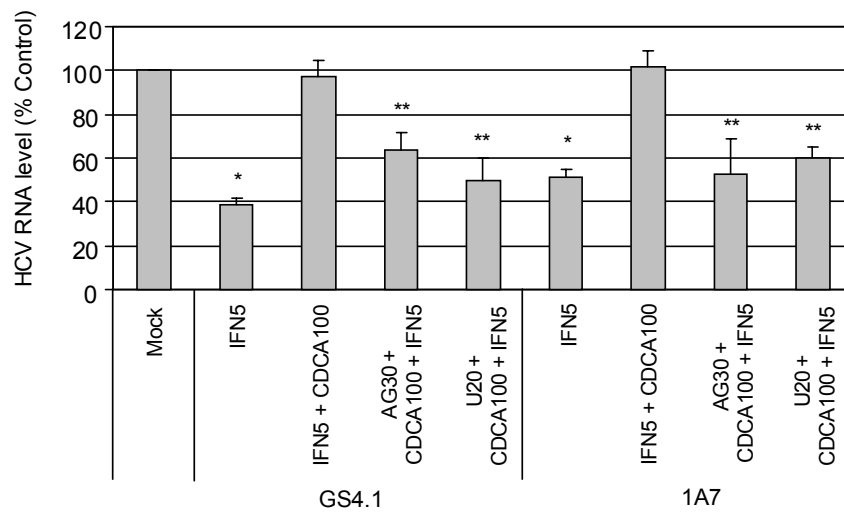
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CHAPTER 4 - The Limitation of Antiviral Effects Against HCV Due to Bile Acids

Abstract

Hepatitis C Virus (HCV) currently poses a significant health problem with the WHO reporting over 170 million infected patients worldwide, with an estimated 3 to 4 million new infections occurring each year. With HCV accounting for almost 66% of the liver transplants in the United States it is a disease that draws considerable attention. There are 6 recognized genotypes of HCV worldwide, with genotypes 1 and 2 being of major concern in the United States. Currently, the standard treatment for chronic HCV infections is a combination of interferon (IFN) and ribavirin, but around half of patients infected with HCV genotype 1 fail to show a sustained virologic response to the treatment and remain chronically infected. We previously demonstrated that bile acids compromise the anti-HCV effects of IFN. It has been shown that 17AAG (17-[Allylamino]-17-demethoxygeldanamycin), cyclosporine A, and ribavirin could reduce the replication of HCV in replicon-harboring cells. In this study, we examined the effects bile acids on anti-HCV activity of these agents in mono- or combined treatment with IFN in replicon-harboring cells containing either genotype 1A or 1B. First, we found that bile acids also compromised the anti-HCV action of each agent. Bile acids either partially or completely blocked the anti-HCV action of 17AAG, cyclosporine A, or ribavirin in the cells. When we examined the effects of bile acids on the combined treatments of each agent with IFN, it significantly restored the anti-HCV activity of each agent with varying degrees. With or without bile acids, 17AAG or cyclosporine A have significantly higher efficiency than ribavirin in mono-

or combined therapy. These results suggest the importance of combination therapies to increase the overall sustained virological response in HCV patients.

Introduction

HCV poses a significant global health problem. The World Health Organization (WHO) estimates that there are approximately 170 million infected individuals worldwide with an additional 3 to 4 million additional infections occurring yearly, resulting in close to 3% of the world's population being chronically infected with HCV (1). Of the 3 to 4 million new infections occurring each year only 25% of these are symptomatic infections. Unfortunately, out of these 25%, 60% to 80% will develop chronic liver disease with approximately 20% of those patients progressing to liver cirrhosis. HCV is one of the leading causes of liver cancer, causing between 50% to 76% of all liver cancer cases and accounting for almost 66% of all liver transplants.

The Hepatitis C Virus (HCV) is a member of the *Flaviviridae* family of viruses along with Yellow Fever Virus, West Nile Virus, Dengue Fever Virus, and classical swine fever viruses. The HCV genome is a 9.6-kb positive sense, single stranded RNA genome that encodes at least 10 individual proteins (9, 19, 20, 23). The 10 individual proteins are encoded into one large poly-protein that's composed of approximately 3000 amino acids. This poly-protein is further cleaved by the NS3-4A serine proteases into the 10 individual proteins (9). HCV displays a liver tropism that is not completely understood. The primary receptor for HCV is thought to be CD81 which is found throughout the body eliminating it as a possible reason for liver tropism (7, 13, 28, 31). A second line of reasoning deals with liver specific cofactors that may be required for

HCV replication. Chang *et al* (2004) described the relationship between porcine enteric calicivirus (PEC) and bile acids. Originally PEC was fastidious in tissue culture, it was later determined though that addition of bile acids allowed PEC to actively replicate in cell culture (5). If the replication of HCV is similar, it may also require a liver specific cofactor to actively replicate. Within the past few years multiple genotypes of HCV have been isolated, and successfully tissue culture adapted. Although the majority of strains remain fastidious in cell culture, there now exist representative virus strains from both genotypes 1 and 2 (23, 39, 44, 45, 47). Replicon-harboring cells containing either the nonstructural genes or the full length HCV genome were established to enhance the our ability to design antivirals and better understand the HCV replication cycles in the absence of tissue culture adapted viruses. These systems are currently available in a wide variety of genotypes providing an excellent base for the screening of multiple antiviral drugs across the spectrum of HCV genotypes (2, 24, 37).

Currently, the accepted treatment for chronic HCV infections involves a combination therapy consisting of high dose pegylated interferon (IFN)-α and Ribavirin (3, 42, 46). Both individually and in combination IFN-α and Ribavirin have been shown to be an effective treatment for against HCV viruses from within genotypes 2 and 3. Genotype 2 and 3 viruses have a sustained virological response rate of over 80% after treatment. Unfortunately, HCV viruses that are members of genotype 1 have a much lower response rate to the combination treatment, with sustained virological responses dropping as low as 40 to 50% (26). Unfortunately, the exact mechanisms that underlie the large number of genotype one non-responders are not completely understood. Over the years a number of antivirals including Cyclosporine A and 17-AAG have been looked at for their anti-HCV activity (18, 30, 38, 41).

These antivirals have been demonstrated to have significant antiviral effects on HCV replication either *in vitro* or *in vivo*. Numerous attempts have being made to identify new antiviral compounds that specifically target either viral proteins or cellular proteins that are required for the viral lifecycle. Currently, the majority of the antiviral development for HCV is targeted towards the NS3-4 serine protease and NS5B RNA-dependent RNA polymerase (10, 22). While targeting these proteins is highly effective in disrupting the viral lifecycle there are difficulties with this approach. Viral targeted antivirals for HCV have to be able to cope with the rapid mutation rates of the HCV genome which frequently leads to resistant mutants (33). The second alternative is the development of antivirals targeting various host factors that are required for the viral replication cycle. Due to the fact that these targets display a much slower rate of evolution there is a lower probability that you will see resistant mutants rapidly developing to counter the antivirals (32).

In our previous study, we demonstrated that bile acids promoted HCV replication and compromised the anti-HCV action of IFN-α using replicon-haboring cells containing HCV genotype 1b (GS4.1 cells) (4). Our study suggested that bile acids may be a reason for low efficacy of IFN based treatment in a large percentage of patients with chronic HCV infections (4). Various bile acids including chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA) all promoted HCV replication and interfered with IFN-α action in the replicon-harboring cells (4). Scholtes *et al*. (34) also demonstrated that bile acids enhanced the replication of genotype 1, but not genotype 2 HCV. They further demonstrated that the enhancement is tied to FXR. Recent research has shown that the epidermal growth factor receptor EGFR inhibitor AG1478 and mitogen-activated

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protein kinase kinase MEK inhibitor U0126 have the ability to partially restore the IFN action even in the presence of bile acids (Patton *et al.* Submitted). Therefore, we hypothesized that bile acids may disrupt the overall antiviral effectiveness of multiple mono-therapies.

In this report, we demonstrate that bile acids can significantly reduce the effectiveness of Ribavirin, 17-AAG, and Cyclosporine A when used a mono-therapies against HCV. When Ribavirin was directly compared to 17-AAG or cyclosporine A, Ribavirin demonstrated significantly lower antiviral activity against HCV *in vitro* in the presence of bile acids. Further, we showed that bile acids can partially disrupt the dual therapies with IFN and each antiviral although the overall levels of HCV RNA and protein are well below mock treatment levels with the dual therapy in the presence of CDCA.

Materials and Methods

Cell Lines and Reagents.

GS4.1 (replicon-harboring cells with the HCV 1b genotype, provided by Dr. C. Seeger at the Fox Chase Cancer Center, Philadelphia, PA), was maintained in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal bovine serum and 0.5 μg/ml concentrations of G418 (Cellgro, Manassas, VA). Antibodies against HCV NS5b and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Chenodeoxycholic acid (CDCA), 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG), Cyclosporine A, Ribavirin, and IFN-α (recombinant IFN-α A/D human) were obtained from Sigma-Aldrich (St. Louis, MO). CDCA, 17-AAG, and cyclosporine A were resuspended in dimethyl sulfoxide (DMSO). IFN-α and Ribavirin were suspended and diluted in ddH$_2$O.

Treatment of Replicon-Harboring Cells with Bile Acids, Antivirals, and IFN-α.

First, we examined the cytotoxic effects of each bile acid and/or the inhibitor on GS4.1 or 1A7

cells using a CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI) to

obtain the concentration of each agent with minimum cell toxicity. To examine the antiviral

benefits of each compound GS4.1 replicon-harboring cells were treated for 24 hr with varying

concentrations of Ribavirin (80 or 40 µM), 17-AAG (0.5 or 0.2 µM), or cyclosporine A (1 or 0.5

µM) for 24 hr.   Next the GS4.1 cells were treated with each antiviral at the above concentrations

in the presence of 100 µM CDCA for 24 hr. Finally, each compound was evaluated as a

combination therapy with IFN-α (5U/ml) both with and without CDCA (100 µM). After 24 hr of

treatment each sample was measured for HCV RNA and protein levels using real-time

quantitative reverse transcription-PCR (qRT-PCR) and western blot analysis as described below.

Detection of HCV RNA and NS5b Protein.

The HCV RNA and NS5b protein levels were measured 24 hr after semi-confluent GS4.1 were

treated with CDCA, IFN-α and/or CDCA, and/or one of the antiviral compounds (17-AAG,

Cyclosporine A, Ribavirin) by qRT-PCR or western blot analysis, respectively. RNeasy Mini

Kit (Qiagen, Valencia, CA) was used to isolate total RNA was from GS4.1 cells in 6-well plates

following treatment. After the isolation of the total RNA qRT-PCR was performed using a One-

Step Superscript III qRT-PCR kit (Invitrogen) with primers and probes previously described

(Chang and George, 2007). Western blot analysis was performed on cells lysed with lithium

dodecyl sulfate (LDS) sample buffer containing 2% β-mercaptoethanol. NS5b protein was

detected using a mouse monoclonal anti-NS5B antibody at a 1:1000 dilution in 2% non-fat dry

milk (NFDM) followed by a secondary HRP conjugated goat-anti-mouse (Pierce Biosciences,

Rockford, IL) at 1:1000. Protein was visualized using a chemiluminescent substrate (Pierce

Biosciences). β-actin levels were measured by qRT-PCR and Western blot analysis for the

normalization of RNA and protein levels of each sample, respectively.

Statistical Analysis.

All experiments were repeated a minimum of three separate times. Statistical analysis was

performed using GraphPad Prism 5.0.

Results

Bile Acids Significantly Impaired Mono-Therapy Ttreatments Consisting of Ribavirin, 17-AAG, and Cyclosporine A.

In agreement with previous literature we determined that each of the mono-therapies

significantly reduced the overall levels of HCV RNA when compared to mock treated cells

(Figure 4-1). Further we demonstrated that bile acids significantly reduced the effectiveness of

each of the mono-therapies. Ribavirin reduced the RNA level to 53% ± 8% at a concentration of

80 µM and did not significantly reduce the RNA levels at 40 µM ($p<0.05$) (Figure 4-1A). In the

presence of 100 µM CDCA the effectiveness of Ribavirin was significantly decreased to 132% ±

24% or 187% ± 41% of mock respectively ($p<0.05$) (Figure 4-2A). Replicon-harboring cells

treated with 5U of IFN-α for 24 hr had HCV RNA level of 66% ± 6% when compared to mock

treated cells. In the presence of 100 µM of CDCA the 5U of IFN-α did not significantly reduce

the HCV RNA level, 97% ± 8% ($p<0.05$), when compared to mock treated cells (Patton *et al*, Submitted). The antiviral 17-AAG reduced the HCV RNA level to 52% ± 12% of mock at 0.5 µM or 75% ± 13% of mock at 0.2 µM concentrations ($p<0.05$) (Figure 4-1B). When the cells were treated with 100 µM CDCA and 17-AAG the HCV RNA levels significantly increased to 93% ± 9% or 133% ± 9% respectively (Figure 4-2B) ($p<0.05$). Cyclosporine A reduced the RNA levels to 48% ± 5% using a concentration of 1 µM and 69% ± 8% at a concentration of 0.5 µM (Figure 4-1C). Similar to 17-AAG, when GS4.1 cells were treated in combination with 100 µM CDCA there was a significant decrease in the effectiveness of the cyclosporine A treatment. Replicon-harboring cells treated with a concentration of 1 µM or 0.5 µM of Cyclosporine A and 100 µM of CDCA showed significant increases in the HCV RNA levels from 48% ± 4% or 69% ± 8% after mono-therapy to 78% ± 13% and 135% ± 9% respectively after a 24 hr incubation period ($p<0.05$) (Figure 4-2C). Western blot analysis further supported the qRT-PCR results demonstrating that the HCV NS5b protein levels correlated well with the HCV RNA level in the replicon-harboring cells for each of the tested antiviral (Figure 4-3A-C).

The Bile Acid Mediated Interference in the Anti-HCV Activity of Each Mono-Therapy Can be Partially Mitigated Using Dual Treatments of Ribavirin, 17-AAG, or Cyclosporine A in Combination with IFN-α.

To examine the role of each antiviral compound and its ability to reduce the viral load in the presence of IFN and CDCA each of the 3 antiviral compounds were added in combination with CDCA and IFN-α. The HCV RNA levels of GS4.1 cells treated with a combination therapy consisting of 80 µM or 40 µM of Ribavirin, 100 µM of CDCA, and 5U of IFN were 73% ± 2% or 82% ± 6% respectively($p<0.05$). The 80 µM dose of Ribavirin significantly decreased the

HCV RNA levels when compared with the combination therapy of CDCA and IFN alone (Figure 4-2A). The combination treatments consisting of 17-AAG at 0.5 µM or 0.2 µM resulted significantly decreased RNA levels of 55% ± 3% and 65% ± 2% respectively($p<0.05$) (Figure 4-2B). The triple therapy incorporating Cyclosporine A also resulted in significant decreases of HCV RNA levels, 50% ± 3% or 63% ± 5%, at concentrations of 1 µM or 0.5 µM ($p<0.05$) (Figure 4-2C).

Discussion

Hepatitis C Virus has a liver specific tropism that is not completely understood. In our previously published work we demonstrate that bile acids, both conjugated and unconjugated, can play a role in the up-regulation of HCV RNA and protein levels in both GS4.1 and 1A7 cells (4). We further showed that bile acids have the ability to interfere with the anti-viral activity of IFN-α in a dose dependant manner (4). HCV resides and replicates in the liver where bile acid levels can reach up to 80 µM (21), we have previously suggested that this may play a role in the many genotype one patients who do not respond to IFN-α treatment (4). Here we tested the ability of bile acids to reduce antiviral effectiveness of 3 different antiviral compounds that interact either with the host system or interfere with viral replication. Scholtes et al. (34) has shown that the enhancement of HCV by bile acids occurs through the activation of FXR. The bile acids enhancement of HCV through FXR may indirectly cause a decrease in the antiviral efficacy of multiple mono-therapies (34). We showed that bile acids significantly interfere with multiple mono-therapies we propose the addition of a separate compound to the current treatment regime may significantly increase the effectiveness of the current IFN-α and Ribavirin therapy improving the number of patients exhibiting a sustained virological response.

Ribavirin is an anti-viral drug that is used to treat multiple viral infections including HCV, Human immunodeficiency virus (HIV), and severe respiratory syncytial virus RSV infections (14, 16, 36). Once Ribavirin has been metabolized the altered chemical structures resembles that of a purine molecule. This has lead some to suggest that its mechanism of action may revolves around inducing rapid and terminal mutations after its incorporation in to the replicating viral genome (8). Unfortunately, the mechanism of actions has yet to be conclusively demonstrated and multiple proposed mechanisms exist (11). In a recent clinical study patients with genotype 1 demonstrated a 16% higher sustained virological response when given a combination of IFN-α and Ribavirin, as opposed to IFN-α alone (15). In agreement with literature, as a mono-therapy Ribavirin was able to significantly reduce the HCV RNA levels to 53% when compared to mock treated cells at a concentration of 80 μM although no significant reduction could be seen at 40 μM. In the presence of CDCA Ribavirin was unable to reduce the overall HCV load to below the mock level (132% $\pm$ 24%) although there was a significant reduction from CDCA treated cells at 80 μM (Figure 4-1A). This finding is similar to the CDCA mediated interference with IFN-α activity that was reported by Chang et al (2008). This suggests that the enhancement of the HCV RNA and protein levels after treatment with CDCA are greater than Ribavirin alone can overcome. As a possible solution to this problem we looked at the ability of CDCA to reduce the effectiveness of a dual treatment with IFN-α and Ribavirin. This reduction in antiviral effectiveness was also noted after the addition of CDCA (100 μM) to a dual treatment of Ribavirin (80 μM) and IFN-α (5U / ml), although there was still a significant reduction in the overall HCV RNA levels when compared to mock treated cells [(46% $\pm$ 2%, 57% $\pm$ 2% (without CDCA) or 73% $\pm$ 2%, 82% $\pm$ 6% (with CDCA)) 80 μM and 40 μM respectively] (Figure 4-2A).

One of the current draw backs to the treatment of HCV patients with Ribavirin is the high drug concentrations that are necessary to have any significant effect of the replication of HCV. At high concentrations the primary side effect of Ribavirin is haemolytic anemia, which has been reported in 13% of all clinical trials.

Cyclosporine A is an immunosuppressive drug that is known to have anti-HCV effects . It has been demonstrated to bind to group B cyclophilins which are required by the HCV replicase complex for viral replication. Cyclosporine D, a non-immunosuppressive variant of A appears that appears to have similar abilities to inhibit the replication of HCV by binding to types B cyclophilins (29, 30). Currently, a variety of cyclosporine derivatives are being tested as candidate antiviral HCV drugs, with the major benefit of many of these derivatives being their non-immunosuppressive nature. Three of these derivatives Debio-025, NIM811, and Valisporivir have both shown some promising advances in the developments of anti-HCV drugs that target the host system and are in phase III clinical trials (6, 25, 40). Cyclosporine A has been studied extensively as an HCV antiviral and been shown both in vitro and in vivo to have strong anti-HCV effects. As a mono-therapy Cyclosporine A proved effective at a low concentration, 1 µM and below, at significantly reducing the HCV RNA levels (Figure 4-1B). When cells were treated with a combination of CDCA 100 µM and Cyclosporine A there was a significant increase in HCV RNA levels when compared to the mono-therapy alone [48% ± 4%, 69% ± 8% (without CDCA) or 78% ± 13%, 135% ± 9%(with CDCA)) 1 µM and 0.5 µM respectively] (Figure 4-1B). Unlike Ribavirin and CDCA treated cells, at 1 µM there was still a reduction after CDCA treatment when compared to mock treated cells. When CDCA was added to the dual treatment consisting of Cyclosporine A and IFN-α there was a slight reduction in the

overall effectiveness of the dual therapy, but a significant reduction over the mock treated cells was still shown [(34% ± 4%, 52% ± 2% (without CDCA) or 50% ± 3%, 63% ± 5% (with CDCA)) 1 µM and 0.5 µM respectively] (Figure 4-2B). Cyclosporine A has been used in multiple clinical trials as an antiviral treatment for HCV, but due to the immunosuppressive properties of the molecule it has not been added to any of the current treatment regimes (12). Many of the current derivatives including Debio-025 and NIM-811 may significantly enhance the overall effectiveness of the standards treatment practices. These derivatives have the anti-HCV properties of cyclosporine A but lack its immunosuppressive properties that make it problematic for use in the treatment of patients.

17-AAG a derivative of geldanamycin is a potent HSP90 inhibitor (17, 27, 35). The geldanamycin family was originally isolated as an antibiotic and display weak antibiotic properties. 17-AAG is currently being actively studies for the treatment of multiple types of cancer due to its ability to inhibit HSP90 which results in the breakdown of specific cellular proteins that may cause the death of multiple types of cancer cells (43). In January of 2009 Ujino et al. (38) published a paper demonstrating that the ability of 17-AAG to inhibit HSP90 also lead to the degradation of NS3, an HCV protein that directly interacts with HSP90. The degradation of NS3 occurred in a dose dependent manner and led to the suppression of HCV replication (38). Our results further confirm the ability of low doses of 17-AAG, 0.5 µM and 0.2 µM, significantly reduce the HCV RNA levels in replicon-harboring cells (Figure 4-1C). To further elicit the effects of bile acids on HCV replication in the presence of the antiviral 17-AAG we treated GS4.1 cells with 100 µM CDCA and either 0.5 µM and 0.2 µM 17-AAG. The results closely mirrored that of the dual treatment with CDCA and cyclosporine A. At a concentration

of 0.5 μM the addition of CDCA decreased the effectiveness of 17-AAG by 41% when both

treatments are normalized to mock. The interference by bile acids was additionally seen after

CDCA was added to the combination treatment of IFN-α and 17-AAG. Although similar to

cyclosporine A there was still a significant reduction in the HCV RNA and protein levels after

the combination treatment when compared to mock treatments [(28% ± 3%, 50% ± 8% (without

CDCA) or 55% ± 3%, 65% ±2% (with CDCA)) 0.5 μM and 0.2 μM respectively] (Figure 4-2C).

These data suggest that bile acids have the ability to interfere with the anti-HCV activity of

multiple different antivirals, and the mechanism of action of the antiviral whether cell based or

viral based plays no role in its depressed antiviral activity. This suggests that the over-

enhancement of HCV levels by bile acids through FXR may overcome the maximum safe dose

of antivirals when used as a mono-therapy. Although the bile acids still effect the dual therapies,

these therapies still significantly reduce the HCV RNA and protein levels. The combination

therapy consisting of IFN-α and Ribavirin is already the standard treatment. Here we describe

the effects of bile acids on two other antivirals cyclosporine A and 17-AAG. Both of these

antivirals provide a significant reduction in HCV levels, and a greater antiviral effect in the

presence of bile acids than the current treatment choice Ribavirin. In the presence of CDCA the

HCV RNA levels in Ribavirin treated cells were elevated above the mock control. Both 17-

AAG and cyclosporine A still showed reduced HCV RNA levels in the presence of CDCA. The

addition of one or the other of these final two compounds that have been shown to display

antiviral activity could improve the current IFN-α based treatments for HCV with the hopes of

increasing the number of patients with a genotype 1 virus that exhibit a sustained virological

response. Our reasoning behind this is the ability of both 17-AAG and cyclosporine A to still

achieve sub-mock HCV RNA levels even in the presence of CDCA.

The current treatment standard consisting of a combination of Ribavirin and IFN-α has been shown to be effective at improving the number of patients exhibiting a sustained virological response. Unfortunately, Ribavirin was one of the poorest performing antiviral compounds in the study, requiring the highest does and only displaying a limited improvement in HCV RNA and protein levels in the presence of CDCA. Both 17-AAG and cyclosporine A showed significantly better reductions of both HCV RNA and protein levels the presence of bile acids. In summary, we demonstrate that bile acids have the ability to reduce the anti-HCV effects of Ribavirin, 17-AAG, and Cyclosporine when used as a mono-therapy. Further, either 17-AAG or Cyclosporine A offered the best combination treatment with IFN-α for HCV reduction in the presence of bile acids.

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Figure 4-1 Reduction of the HCV RNA Levels in GS4.1 Replicon-Harboring Cells After Treatment with Each Antiviral Compound.

Semi-confluent GS4.1 cells were treated with mock-medium, CDCA, 17-AAG, Cyclosporine A, or Lapatinib for 24 hr before the HCV RNA levels were measured by qRT-PCR. A) qRT-PCR levels after treatment with mock-medium, CDCA, IFN, Ribavirin 80 µM (Ribavirin 80), Ribavirin 40 µM (Ribavirin 40), Ribavirin 80 and CDCA, or Ribavirin 40 and CDCA for 24 hr. B) qRT-PCR levels after the treatment with mock medium, CDCA, IFN, 17-AAG 0.5 µM (17-AAG 0.5), 17-AAG 0.2 µM (17-AAG 0.2), 17-AAG 0.5 and CDCA, or 17-AAG 0.2 and CDCA for 24 hr. C) qRT-PCR levels after treatment with mock-medium, CDCA 100 µM (CDCA), IFN-α 5U (IFN), Cyclosporine A 1 µM (Cyclo 1), Cyclosporine A 0.5 µM (Cyclo 0.5), Cyclo 1 and CDCA, or Cyclo 0.5 and CDCA for 24 hr. Asterisk (*) indicates that the RNA levels by the treatment were significantly increased compared to those by control (mock-medium) treatment ($p<0.05$).
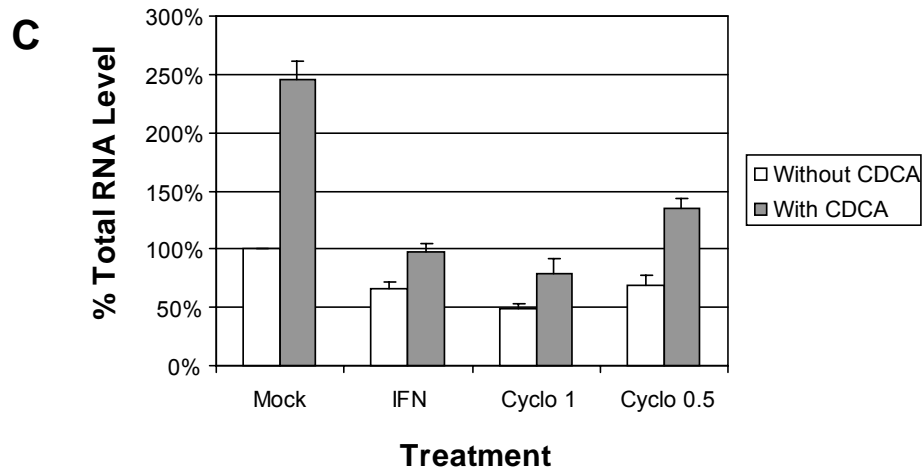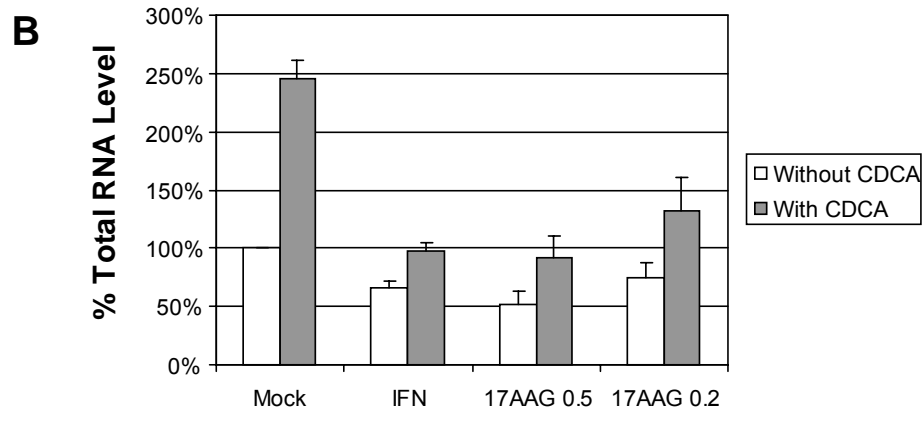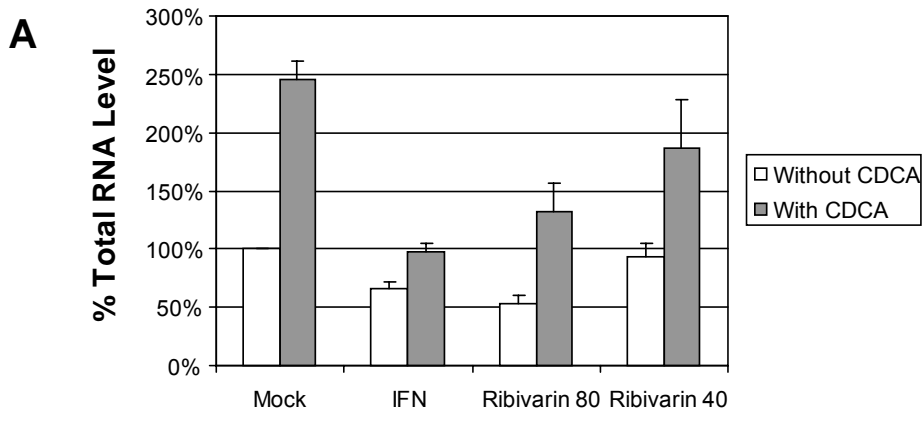
Figure 4-2. Enhancement in the Antiviral Activity of IFN-α in the Presence of CDCA when Combined with a Second Antiviral.

Semi-confluent GS4.1 replicon-harboring cells were treated with mock-medium, CDCA, IFN, CDCA+IFN, or CDCA and IFN and either Ribavirin, 17-AAG, Cyclosporine A, or Lapatinib. A) Real time qRT-PCR analysis of HCV RNA levels after treatment with CDCA 100 μM (CDCA), Ribavirin 80 μM (Ribavirin 80), Ribavirin 40 μM (Ribavirin 40), IFN-α 5U (IFN), CDCA and Ribavirin 80, CDCA and Ribavirin 40, Ribavirin 80 and IFN, Ribavirin 40 and IFN, Ribavirin 80 and IFN and CDCA, Ribavirin 40 and IFN and CDCA for 24 hr. B) Real time qRT-PCR analysis of HCV RNA levels after treatment with CDCA 100 μM (CDCA), 17-AAG 0.5 μM (17-AAG0.5), 17-AAG 0.2 μM (17-AAG0.2), IFN-α 5U (IFN), CDCA and 17-AAG0.5, CDCA and 17-AAG0.2, 17-AAG0.5 and IFN, 17-AAG0.2 and IFN, 17-AAG0.5 and IFN and CDCA, 17-AAG0.2 and IFN and CDCA for 24 hr. C) Real time qRT-PCR analysis of HCV RNA levels after treatment with CDCA 100 μM (CDCA), Cyclosporine A 1 μM (Cyclo1), Cyclosporine A 0.5 μM (Cyclo0.5), IFN-α 5U (IFN), CDCA and Cyclo1, CDCA and Cyclo0.5, Cyclo1 and IFN, Cyclo0.5 and IFN, Cyclo1 and IFN and CDCA, Cyclo0.5 and IFN and CDCA for 24 hr. Asterisk (*) indicates that the RNA levels by the treatment were significantly increased compared to those by control (mock-medium) treatment ($p<0.05$).
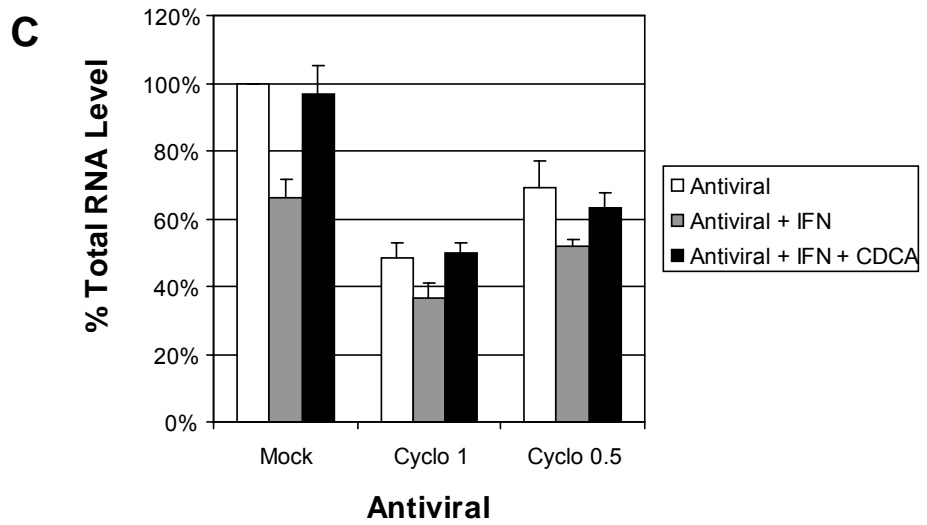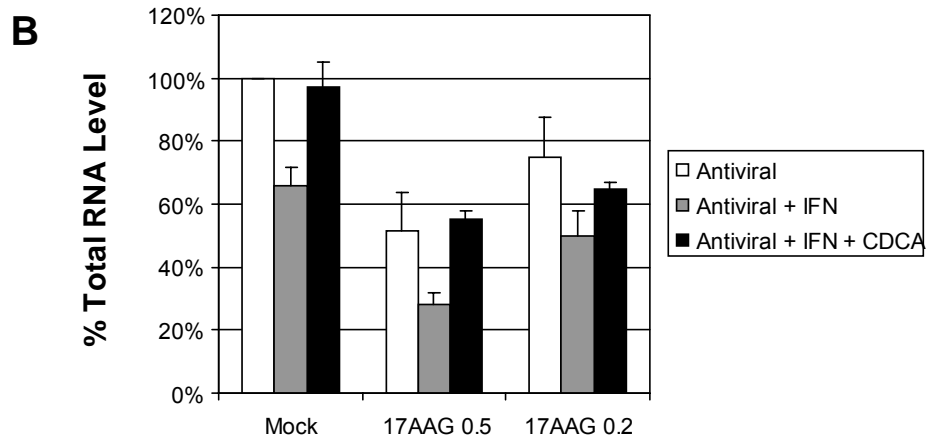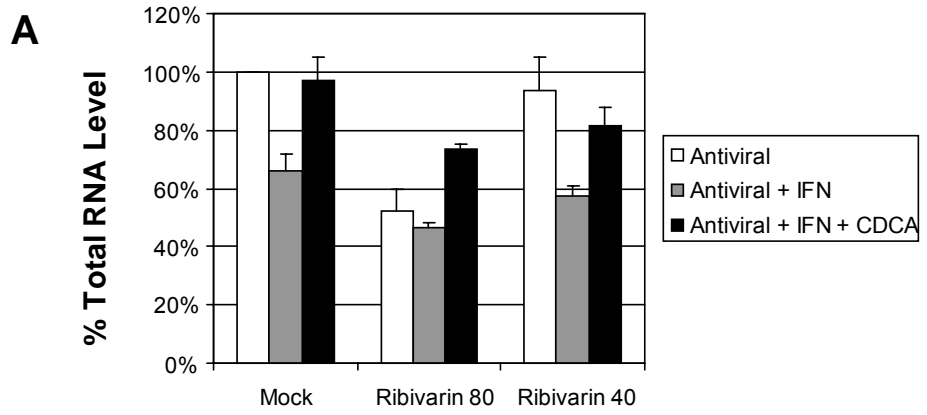
Figure 4-3. Western Blot Analysis of Antiviral Compounds in Combination with IFN-α and CDCA.

Semi-confluent GS4.1 cells were incubated with mock-medium, CDCA, CDCA + IFN, Ribavirin or Cyclosporine or 17-AAG, or a combination of CDCA + IFN + either Ribavirin, Cyclosporine, or 17-AAG for 24 hours. A) Western blot analysis of GS4.1 cells treated with mock-medium (Lane 1), CDCA 100 μM (Lane 2), Ribavirin 80 μM (Lane 3), Ribavirin and IFN 5 U/ml (Lane 4), IFN and CDCA (Lane 5), and CDCA + IFN + Ribavirin (Lane 6). B) Western blot analysis of GS4.1 cells treated with mock-medium (Lane 1), CDCA 100 μM (Lane 2), 17-AAG 0.5 μM (Lane 3), 17-AAG and IFN 5 U/ml (Lane 4), IFN and CDCA (Lane 5), and CDCA + IFN + 17-AAG (Lane 6). C) Western blot analysis of GS4.1 cells treated with mock-medium (Lane 1), CDCA 100 μM (Lane 2), Cyclosporine 1 μM (Lane 3), Cyclosporine and IFN 5 U/ml (Lane 4), IFN and CDCA (Lane 5), and CDCA + IFN + Cyclosporine (Lane 6).

Lane 1
Lane 2
Lane 3
Lane 4
Lane 5
Lane 6

A   Ribavirin 80 µM

B   17-AAG 0.5 µM

C   Cyclosporine A 1 µM
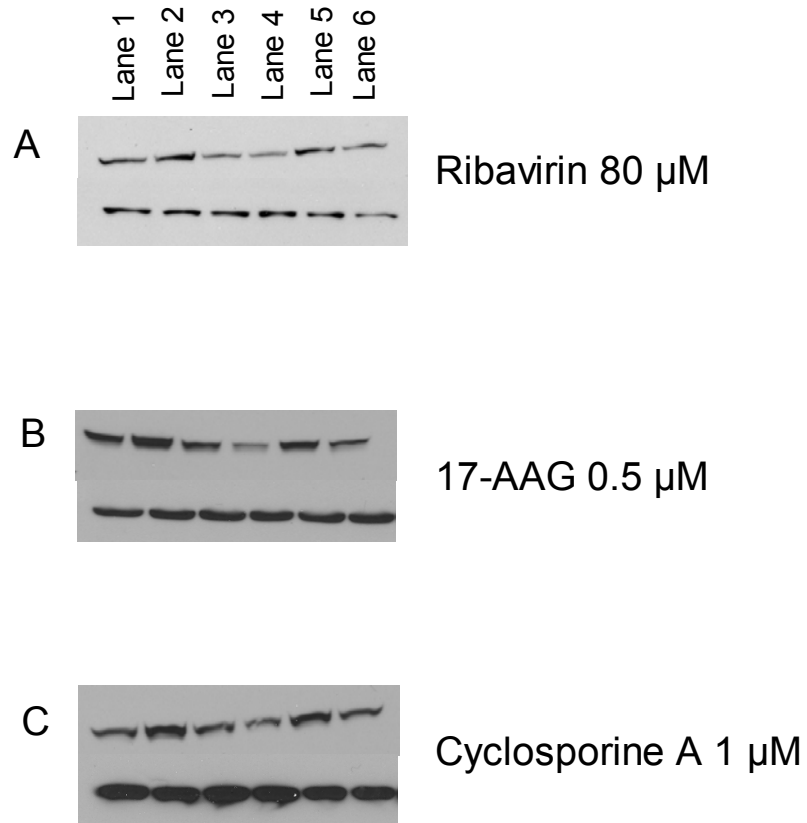
Lane 1: Mock
Lane 2: CDCA 100µM
Lane 3: Antiviral
Lane 4: Antiviral + IFN
Lane 5: Antiviral + CDCA
Lane 6: Antiviral + IFN + CDCA

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CHAPTER 5 - Conclusion and Future Directions

Positive sense RNA viruses comprise a diverse group of human and animal viruses. Their

pathogenicity can range from sub-clinical to lethal and the rapid evolution of this group of

viruses can rapidly create resistant mutants against our current stocks of antivirals. There are

currently a number of human and animal diseases caused by RNA viruses that no efficacious

vaccine exists for. A better understanding about the interactions between these viruses and their

hosts cells will help to design better and more effective treatments to combat these viruses.

The ability of various RNA viruses to disrupt and control a variety of host cellular pathways and

cycles has been well documented. The modulation of various host factors have been shown to

alter the overall replication levels of viruses. The research presented in this dissertation

specifically looks at the modulation of the cell surface receptor CD163 and the role it plays in the

replication of the PRRSV virus, the effects of bile acids on the replication of HCV and how the

disruption of the EGFR and the ERK pathway mitigates this enhancement in replication of HCV

in the presence of bile acids. Finally we studied the ability of bile acids to reduce the overall

effectiveness of 3 different known antiviral compounds to HCV.

We first confirmed Calvert *et al.* initial findings suggesting that CD163 is able to create a

PRRSV permissive cell line with the creation of the LLCPK-163 stable cell lines. Following this

we demonstrated that the expression of CD163 on the cell surface of PAMs, monocyte derived

macrophages, and differentiating monocytes can be altered in the presence of TPA, LPS, and IL-

10. TPA significantly reduced the overall levels of CD163 expression on all three cell types. A

decrease in the overall levels of CD163 after LPS treatment was only observed on the monocyte derived macrophages and differentiating monocytes. An enhancement in the total levels of CD163 was seen after treatment of both monocyte derived macrophages and differentiating monocytes after IL-10 treatments. All cells were infected with 129-GFP and the correlation between CD163 levels and PRRSV replication levels were observed. There was a strong correlation between the expression level of CD163 and overall PRRSV levels, as determined by flow cytometry and $TCID_{50}$. Finally we showed that a monoclonal antibody to CD163 is sufficient to reduce the overall levels of PRRSV infection in PAMs.

The second group of experiments focused on the effects of bile acids on the replication of HCV. Our lab group had previously demonstrated that bile acids could enhance the expression levels of HCV RNA and Proteins in GS4.1 replicon-harboring cells and that this enhancement interfered with the antiviral action of IFN-α. We extended these results by demonstrating that this enhancement was due to the activation of the ERK pathway and by disrupting the activation of the ERK pathway by bile acids using either AG1478 or U0126 we could partially restore the antiviral activities of IFN-α. We further showed that the addition of bile acids to both GS4.1 and 1A7 cells alters the cell cycle profile and the extended S phase that is seen correlates with the increased levels of HCV RNA and proteins.

The final group of experiments furthers the original HCV work looking at the effects of bile acids on the effectiveness of three known HCV antivirals. In each of the three cases we demonstrated that the effectiveness of each antiviral was diminished in the presence of bile acids when used as a monotherapy alone. When the antiviral was combined with IFN-α and enhanced

antiviral effect was seen in the presence of bile acids. We demonstrated that in vitro the current therapy of Ribavirin and IFN-α was actually the least effective of the three combination treatments, and required far higher doses of Ribavirin than were required of cyclosporine A or 17-AAG.

The development of antivirals targeting specific host factors may prove beneficial in combating these different RNA viruses. Further research on the bile acid mediated promotion of HCV replication will be necessary to elicit the overall impact on the current and future treatment regimes. A more detailed study using a genotype 1 tissue culture adapted virus will help us to better understand how the activation of the ERK pathway by bile acids and HCV replication are tied together. Using this information it may be possible to generate a series of treatments that block this promotion and enhance the overall effectiveness of the current HCV treatment protocols.

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