Recombinant Human L-Ficolin Directly Neutralizes Hepatitis C Virus Entry

Running title: L-ficolin neutralizes HCV entry

Mohamed R. Hamed[1,2], Richard J. P. Brown[1†], Carsten Zothner[3], Richard A. Urbanowicz[1], Christopher P. Mason[1], Anders Krarup[4+], C. Patrick McClure[1], William L. Irving[1], Jonathan K. Ball[1], Mark Harris[3], Timothy P. Hickling[1‡] & Alexander W. Tarr[1#]

[1] School of Life Sciences, and Nottingham Digestive Diseases Biomedical Research Unit, University of Nottingham, UK.

[2] Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt

[3] School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, UK

[4] Biochemistry Department, University of Oxford, UK

#Corresponding author: Dr Alexander W. Tarr, Virus Research Group, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH. Tel: 01158230746; Fax: 01158230759; email: alex.tarr@nottingham.ac.uk

†Current address: TWINCORE Zentrum für Experimentelle und Klinische Infektionsforschung GmbH,Feodor-Lynen-Str. 7,30625 Hannover, Germany

+Current address: Crucell, Archimedesweg 4-6, 2333 CN Leiden, Netherlands

‡Current address: Pfizer, Pharmacokinetics, Dynamics and Metabolism, 1 Burtt Road, Andover, MA 01810, USA.

Abstract word count: 155

Main text word count: 3747

Key words:

hepatitis c virus; ficolin; virus entry; neutralization; glycosylation

Abstract

L-ficolin is a liver-expressed soluble pattern recognition molecule that contributes to innate immune defense against microorganisms. It is well described that binding of L-ficolin to specific pathogen-associated molecular patterns activates the lectin complement pathway resulting in opsonization and lysis of pathogens. In this study we demonstrated that in addition to this indirect effect, L-ficolin has a direct neutralizing effect against Hepatitis C Virus (HCV) entry. Specific, dose-dependent binding of recombinant L-ficolin to HCV glycoproteins E1 and E2 was observed. This interaction was inhibited by soluble L-ficolin ligands. Interaction of L-ficolin with E1 and E2 potently inhibited entry of retroviral pseudoparticles bearing these glycoproteins. L-ficolin also inhibited entry of cell-cultured HCV in a calcium-dependent manner. Neutralizing concentrations of L-ficolin were found to be circulating in the serum of HCV-infected individuals. This is the first description of direct neutralization of HCV entry by a ficolin and highlights a novel role for L-ficolin as a virus entry inhibitor.

Introduction

Chronic Hepatitis C Virus (HCV) infection affects approximately 130 million people. Chronic infection is a risk factor for cirrhosis and hepatocellular carcinoma, resulting in the requirement for liver transplantation [1]. Entry inhibitors are an attractive treatment in the clinical setting of liver transplantation, where they might prevent infection of transplanted tissue. Current therapies target the HCV NS3 protease and the NS5B polymerase [2], but cannot prevent initial infection.

Binding of HCV envelope glycoproteins E1 and E2 to cellular CD81 and SR-B1 is essential for HCV entry, and antibodies that inhibit these interactions neutralize entry (reviewed in [3]). These glycoproteins are targets for therapeutic intervention and host immunity [4,5]. They possess up to 5 and 11 N-linked glycosylation sites, respectively [6]. These glycans are structurally heterogeneous [7], and contribute to glycoprotein biosynthesis, modulation of infectivity and evasion of neutralizing antibodies [8,9]. Many glycosylation sites are highly conserved across genetically diverse HCV isolates [10]. This makes them attractive targets for anti-viral drug development. The lectins cyanovirin-N [11], griffithsin [12] and mannose binding lectin [10] all inhibit entry by binding to these glycans.

Ficolins are a family of serum proteins functionally and structurally related to the collectins, sharing quaternary structure with mannose binding lectin (MBL) and complement component C1q [13]. They consist of disulfide-linked 35kDa polypeptides organized into trimers. Oligomers of these three polypeptides form functional dodecamers [13]. They bind glycan-containing pathogen-associated molecular patterns (PAMPs) and

activate the complement cascade. The carbohydrate-binding activity is attributed to the C-terminal fibrinogen-like binding domain that has general specificity for N-acetyl groups on the outer walls of microorganisms [14]. In humans, three ficolins have been identified; L-ficolin, M-ficolin, and H-ficolin [15]. H-ficolin and L-ficolin are expressed by hepatocytes. L-ficolin has a broad binding specificity for targets including galactose, β-glucan, acetylated compounds, N-acetylglucosamine and N-acetylcysteine. Binding is mediated by four binding sites in the C-terminal fibrinogen-like binding domain, some of which require calcium for interaction [16]. L-ficolin associates with MBL-associated serine proteases (MASPs), resulting in complement activation, phagocytosis and clearance of pathogens bearing N-acetylated structures such as N-Acetyl glucosamine (GlcNAc) [17,18], a major component of bacterial cell walls that is also incorporated into virus glycoproteins. This early innate recognition may play a critical step in priming adaptive immune responses to infection [19].

The contribution of ficolins to controlling virus infections is poorly understood. Porcine ficolin-α reduces infectivity of porcine reproductive and respiratory syndrome virus (PRRSV) [20]. Human L-ficolin also inhibits infectivity of Influenza A *in vivo* [21]. A role for L-ficolin in immune recognition of HCV virions may exist, as binding of a monomeric recombinant L-ficolin to the HCV envelope glycoproteins has been demonstrated to activate the complement cascade resulting in cell lysis [22]. However, no direct antiviral effect on HCV has been described for L-ficolin. Here, the ability of a purified recombinant oligomeric L-ficolin to directly neutralize HCV was investigated. Interaction of L-ficolin with recombinant HCV envelope glycoproteins in the context of HCV pseudoparticles

(HCVpp) and cell cultured HCV particles (HCVcc) was found to result in direct inhibition of virus entry.

Materials and Methods

Purification of plasma L-ficolin. L-ficolin was isolated from citrated human plasma, using GlcNac-Sepharose-4B beads according to the method of Cseh et al. [23].

Expression of recombinant FLAG-tagged L-Ficolin: Human L-ficolin cDNA was amplified from an IMAGE clone BCO 69572 (Open Biosystem) to create a recombinant amino-terminal FLAG-tagged L-ficolin (primers available upon request). The modified L- ficolin was cloned into pcDNA-DEST26 (Invitrogen) and expressed in HEK 293T cells cultured in DMEM. Culture supernatants were clarified and L-ficolin purified using anti-FLAG M2 affinity resin (Sigma). L-ficolin was eluted with 175 μgmL^{-1} FLAG peptide. Fractions were collected and dialyzed with TBS (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl). Protein separation was performed on a 10% SDS-PAGE gel, followed by staining with SimplyBlue (Life Technologies). Western blotting was performed on separated proteins with anti-L-ficolin mAb GN5 (Hycult) or anti-FLAG mAb (Sigma). Total protein concentration was determined using a Bicinchoninic Acid (BCA) assay (Pierce), while active L-ficolin was measured using an acetylated-BSA binding assay (supplementary methods).

L-ficolin HCV glycoprotein binding assay and inhibition. HCV glycoproteins E1/E2 and 6xhis-tagged soluble E2 constructs were described previously [24,25]. Maxisorp plates were coated with anti-L-ficolin antibody GN5 (Hycult) in PBS and incubated at 4^oC overnight. Plates were blocked with PBS-Tween, 5% milk and incubated with 5μg/mL L-ficolin. Lysates containing HCV glycoproteins E1/E2 derived from genotype 1 (H77c; Accession number AF011751), diluted 1⁄10 in PBS–Tween, or purified sE2 (4 μg/mL) in

PBS-Tween were added. A cell lysate from untransfected 293T cells was included as a negative control. After washing, 1µgmL^{-1} biotinylated mAb AP33 [26] was added for 1 hour. After washing three times, wells were incubated for 30 minutes with 0.5 µgmL^{-1} HRP-conjugated streptavidin. Binding was detected at 620nm after incubation with Tetramethylbenzidine (Sigma). Inhibition experiments were performed with an additional step after L-ficolin incubation, adding 100µL/well of serial dilutions of either GlcNAc, CysNAc, or D-Mannose. After 1 hour, the plates were washed three times before addition of HCV glycoproteins.

Neutralization of pseudovirus entry. HCV pseudoparticles (HCVpp) were prepared as previously described [27] incorporating E1/E2 glycoproteins from HCV genotype 1a (H77c; AF011751), 2a (JFH1; AB047639), 3a (UKN3A13.6; AY894683) and genotype 4a (UKN4.11.1; AY734986). Pseudoparticles possessing the VSV G protein were also produced. Infectivity of purified pseudoparticles was assessed using Huh7 cells, incubating particles with purified L-ficolin preparations (quantified by either BCA assay or functional binding assay) for one hour in Dulbecco's Modified Eagle Media (DMEM) before addition to target cells. A fraction from the L-ficolin purification process containing no detectable protein (determined by BCA assay) was used as a control. Luciferase activity was assessed after 72 hours. Neutralization assays with HIV-1 pseudoviruses were performed essentially in the same manner, using glycoproteins from strain HXB2. Infection of TZM-bl cells was performed as previously described [28]. To assess any effect of L-ficolin on Huh7 cells, fractions containing L-ficolin oligomers (or controls) were incubated with cells at 4ºC for one hour prior to washing with PBS and addition of pseudoviruses.

Neutralization of cell cultured HCV (HCVcc) infection. Neutralization assays with JFH1 HCVcc were performed as previously described [27], using similar conditions to those of HCVpp assays. Neutralization was performed with 100FFU of virus, and different concentrations of purified L-ficolin protein. The effect of calcium was determined using an approximate IC_{50} concentration of oligomeric L-ficolin in the presence of 2mM or 7mM $CaCl_2$. Infection was determined by staining for the presence of HCV NS5A using antibody 9E10 [29]. Neutralization was calculated by as a percentage of an uninhibited control.

Statistical analysis. Unpaired t tests or one-way ANOVA tests were used as appropriate to determine differences between mean binding/neutralization values. Serum concentrations of L-ficolin in different groups of individuals were compared using a Mann-Whitney U test.

Results

In vitro expressed recombinant L-ficolin forms oligomers similar to serum-purified L-ficolin. Natural L-ficolin exists as oligomers of a 35kD subunit, up to and including a dodecamer form (Figure 1A). These oligomers were observed for fractions of L-ficolin purified from human serum after passage through a GlcNAc-Sepharose matrix and elution with soluble GlcNAc (Figure 1B, left panel). Proteins observed at 35 kDa, 70 kDa, and above 250 kDa represented monomers, dimers, and higher order oligomers, respectively. Consistent with previous reports [30], proteins were also observed at molecular masses greater than the expected dodecamer, suggesting that L-ficolin is able to form covalently-linked higher molecular weight oligomers. Under reducing conditions the oligomers of serum L-ficolin were reduced to a molecular mass of 35 kDa (Figure 1B, right panel), with traces of dimer or trimer. Recombinant FLAG-tagged, affinity purified L-ficolin was observed as a similar mixture of monomers and oligomers, both when probed with anti-L-

ficolin mAb (Figure 1C), and with anti-FLAG mAb (Figure 1D), indicating that the recombinant protein possesses similar structure to the *in vivo* form. Under these non-reducing conditions, higher oligomers including a possible dodecamer complex were observed. Treatment with dithiothreitol resulted in only the monomeric recombinant protein resolved by western blot (Figure 1E).

Recombinant L-ficolin interacts with HCV glycoproteins. Two recombinant glycoprotein constructs derived from the HCV infectious clone H77c [31] were used to model the interaction between L-ficolin and HCV virions. Binding of both recombinant L-ficolin and L-ficolin purified from serum to the E2 glycoprotein ectodomain (aa363-661) was comparable (Figure 2A). This was confirmed by dose-dependent binding of recombinant L-ficolin to E1/E2 heterodimers (aa170-746) (Figure 2B).

Pre-incubating L-ficolin with ligands resulted in competition for E2 binding (Figure 2C). Both N-acetyl-cysteine (CysNAc) and GlcNAc inhibited the binding interaction between recombinant L-ficolin and the E2 ectodomain in a dose-dependent manner. Fifty per cent inhibition was achieved with 1.8 mM CysNAc, and 260 mM GlcNAc. At the greatest concentration tested (1 M), the MBL ligand D-Mannose had no effect on binding.

L-ficolin neutralizes genetically diverse HCV strains. Having demonstrated an interaction between recombinant L-ficolin and the HCV glycoproteins, the effect of L-ficolin on entry of HCVpp possessing the HCV glycoproteins isolated from patient viruses was investigated. Before performing these experiments the expression of L-ficolin by target Huh7 cells was assessed. No cellular expression of L-ficolin in the target cells was

observed, either by immunofluorescence or by quantitative RT-PCR of L-ficolin mRNA (data not shown). Glycoproteins derived from genetically diverse HCV viruses were tested in a pseudoparticle entry assay (Figure 3A). Active L-ficolin in the preparation was quantified using a functional assay binding to acetylated-BSA. At a concentration of $1\mu gmL^{-1}$ active protein almost complete inhibition of entry of pseudoparticles reconstituted with strains H77 (genotype 1a), UKN3A13.6 (genotype 3a) and UKN4.11.1 (genotype 4) was observed (Figure 3A). A control fraction from the purification procedure containing no L-ficolin (as determined by western blot and BCA assay) had no inhibitory effect when diluted equivalently. Entry of pseudoparticles bearing VSV G was also neutralized by L-ficolin, but this L-ficolin preparation had no effect on entry of retroviral pseudoparticles bearing HIV glycoproteins into CD4+/CCR5+ TZM-bl cells, eliminating the possibility of non-specific toxicity (Figure 3A). Recombinant L-ficolin was further separated into three fractions containing a mixture of oligomers/monomers, or only monomers, as analyzed by western blot (Figure 3B) and stained SDS-PAGE (Figure 3C). Consistent with the greater sensitivity of western blotting, protein was only observed in sample F1 in the stained gel. No contaminating protein was observed. Total protein was quantified in these samples by BCA assay. Samples F1, F2 and F3 possessed $157\mu gmL^{-1}$, $46\mu gmL^{-1}$, and $10\mu gmL^{-1}$ protein, respectively. Each sample was diluted to $1\mu gmL^{-1}$ total protein and assessed for neutralizing potency against HCVpp possessing the glycoproteins from strain JFH1 (Figure 3D). Fraction 1 neutralized HCVpp entry by >60%. Fractions 2 and 3 demonstrated no significant inhibition. A negative control fraction possessed no neutralizing activity. To exclude the possibility that the small neutralizing effect of fractions 2 and 3 might be due to the presence of residual FLAG peptide in the eluted material, neutralization experiments with this peptide demonstrated no effect on the entry of HCV pseudoparticles (not shown).

Given that the negative fraction was treated identically and had no effect on entry it is most likely that the neutralizing effect was due to small quantities of oligomeric L-ficolin in these fractions. The sample possessing detectable oligomeric L-ficolin consistently had significantly greater neutralizing effect than any other sample tested. This sample was further found to inhibit the entry of cell-cultured HCV (HCVcc) of strain JFH-1. Sample F1, at a stock of 157μgmL^{-1} was serially diluted in parallel with the negative fraction before incubation with HCVcc. Neutralization by F1 was dose-dependent, with an EC_{50} of 1.2μgmL^{-1} (Figure 3F), while no inhibition was observed for the negative fraction.

To eliminate the possibility that the observed neutralizing effect was a result of L-ficolin interacting with the target cells, pre-incubation experiments were performed with a preparation of L-ficolin. Neutralization was only observed when pseudoparticles were pre-incubated with the L-ficolin-containing sample (Figure 4).

L-ficolin neutralizes cell cultured HCV in a calcium-dependent manner. L-ficolin binding has been described to be partially calcium-dependent [32]. To determine if this is the case for recognition of HCV, neutralization experiments were performed using the HCVcc in the presence of different concentrations of $CaCl_2$. HCVcc particles were prepared in media containing a basal level of 2mM $CaCl_2$, or media supplemented to a final concentration of 7mM $CaCl_2$. Using 1.6μgmL^{-1} L-ficolin in this assay, enhanced neutralization was observed with increasing calcium chloride concentration, indicating that this activity is, at least in part, calcium-dependent. This is likely to be caused by interactions between the S1 binding site and the GlcNAc present on the surface of HCV particles. When L-ficolin treated with 7mM $CaCl_2$ was analyzed by western blot, no

difference in patterns of oligomerization was observed (Figure 5B). Consistent with previous reports [30], the action of calcium is likely to be on binding activity, rather than disulphide-mediated oligomerization.

Inibition of HCV entry by L-ficolin ligands. To further assess the binding specificity to the observed neutralization, inhibition experiments with GlcNAc and CysNAc were performed. At the concentrations demonstrated to inhibit interaction of E2 with L-ficolin, both GlcNAc (data not shown) and CysNAc (Supplementary Figure 1) were also found to inhibit entry in the absence of L-ficolin. As such, any blocking effect of L-ficolin neutralization was not resolvable. This unexpected result could be evidence that cellular receptors involved in HCV entry recognize similar acetyl-containing molecular entities as the ficolin, so that acetyl-containing inhibitors (GlcNAc and CysNAc) also competitively inhibit these interactions. To investigate this further, CysNAc was incubated with either HCV, VSV or HIV-1 pseudoviruses, or target cells, prior to infection (Supplementary Figure 1B). Treatment of both HCVpp and VSVpp with CysNAc resulted in reduction in infectivity, but no effect on HIV-1 entry was observed. It is possible that VSV and HCV share interactions with receptors that interact with this ligand. Unexpectedly, treatment of target cells with CysNAc resulted in a significant increase in entry of HIV-1pp, but not HCVpp or VSVpp. This finding is worthy of further investigation, and may be linked to the plasma-membrane fusion event of HIV-1 compared to the endosomal fusion of VSV and HCV. The lack of inhibition of pseudovirus entry with CysNAc pre-treated cells suggests that this blocking effect is mediated on the virus, rather than the cells, and is not attributed to cell cytotoxicity. Visual inspection of Huh7 cells treated with CysNAc (Supplementary figure 1C) confirmed that no cell death occurred in treated cells, but revealed a visible

change in cell morphology and size, which may be linked to altered receptor expression and resistance to HCV entry.

L-ficolin neutralizes HCV entry at physiologically relevant levels. As many proteins can inhibit viruses at high concentrations, we next determined if the concentration of L-ficolin that resulted in *in vitro* neutralization was physiologically relevant in both healthy donors and a HCV-infected cohort of patients. Serum L-ficolin was quantified by ELISA in the sera taken from healthy donors and patients with chronic HCV infection (Supplementary Figure 2). There was no significant difference between the median L-ficolin concentrations in healthy donors (4.6μg mL-1 S.D. ±1.5) or chronic HCV infections (4.2μg mL-1 S.D. ±1.9) (Mann-Whitney U test, p=0.29). This confirmed that the concentration of L-ficolin that neutralizes HCV entry *in vitro* is biologically relevant, and that individuals with chronic HCV infection do not have impaired capacity to produce L-ficolin.

Discussion

The soluble innate immune effector L-ficolin has been implicated in the control of a range of infectious diseases, acting as an opsonin and activating complement upon binding to glycosylated targets [33]. Here we demonstrated that a recombinant, oligomeric L-ficolin mediates direct neutralization of HCV entry.

A key advance was the expression of correctly-folded oligomeric recombinant L-ficolin in human cells, in contrast to previous studies using bacterially-expressed protein that yielded only monomers [22]. The monomeric form of L-ficolin was described to activate the

285 complement cascade and facilitate complement-mediated lysis of HCV infected cells, but
286 not to inhibit HCV entry. The N-terminal FLAG-tag used for purification had no significant
287 effect on the oligomerization of the recombinant L-ficolin polypeptides, and this oligomer
288 possessed binding equivalent to serum-purified protein. This construct provides a useful
289 tool for further investigations of the direct anti-viral properties of L-ficolin.
290
291 Acetylated sugars are defined ligands for L-ficolin [14]. It is likely that the high mannose
292 oligosaccharides present on the surface of E1/E2 possessing a $GlcNAc_2$ stem are binding
293 targets for L-ficolin [34,35]. There is evidence that two of the N-linked glycosylation sites
294 might possess complex glycans containing terminal GlcNAc residues at residues 423 and
295 430 [7]. These asparagines have been implicated in the entry of HCVpp [8], and HCVcc
296 [9], respectively. The neutralization of entry of HCVcc and HCV pseudoparticles
297 representing HCV genotypes 1, 2, 3 and 4 is consistent with a role for conserved E1/E2
298 glycans in HCV entry. The genotype 3a clone used in this study has previously been
299 described to be resistant to neutralization by broadly-neutralizing antibodies [27,36]. L-
300 ficolin effectively neutralized this isolate, indicating possible therapeutic application for
301 inhibiting entry of antibody neutralization-resistant HCV strains. Consistent with previous
302 reports demonstrating interaction of L-ficolin with GlcNAc [17] and CysNAc [32], both
303 ligands inhibited interaction of recombinant L-ficolin with glycoprotein E2, suggesting
304 interaction of the fibrinogen-like domain and N-linked glycans. Four discrete binding sites
305 in the L-ficolin fibrinogen-like domain have been identified (S1-4), which possess different
306 binding specificities [37]. This may account for the difference observed in competition
307 assays with the ligands GlcNAc and CysNAc; although they both bind in the S2 site,
308 GlcNAc also binds around the S1 site, while CysNAc is able to bind to site S3, and at high

309 concentrations, to other sites with little structural interaction [37]. The unexpected
310 discovery that both GlcNAc and CysNac inhibited HCV entry *in vitro* also raises new
311 questions about the nature of virus-cell interactions. It is possible that these ligands alter
312 expression of host receptors, and is worthy of further investigation.
313 The calcium-dependent nature of the neutralizing activity suggested that the accessibility of
314 the binding site on L-ficolin is modulated by the presence of a Ca^{2+} ion as previously
315 described [16]. As the fibrinogen-like domain possesses multiple binding sites with
316 differential specificities, the exact interactions between the HCV glycoproteins with the
317 binding surface of L-ficolin remain to be determined. We also eliminated the possibility
318 that L-ficolin interactions with target cells make them refractory to virus entry,
319 demonstrating a virion-specific effect. The exact molecular interaction between L-ficolin
320 and E1/E2 is currently under investigation.
321
322 Recombinant oligomeric L-ficolin inhibited entry of both HCV and VSV pseudoviruses. It
323 is still not understood what common factor determines this sensitivity to L-ficolin
324 neutralization, but this molecule has potential for application as an entry inhibitor for other
325 enveloped viruses. It is plausible that neutralization specificity is associated with specific
326 modifications to carbohydrates on the glycoprotein surface. This neutralizing activity is
327 consistent with the ability of porcine ficolin-α to bind and neutralize PRRSV. Ficolin-α
328 reduced cytopathic effect of PRRSV and inhibited replication of infectious viral particles
329 [20]. However, soluble lectins have also been demonstrated to inhibit antibody
330 neutralization of Human Immunodeficiency Virus 1 [38], suggesting that complex
331 interactions might take place at the surface of the virion.
332

It remains to be demonstrated if binding of this oligomeric L-ficolin construct to HCV might result in secondary anti-viral properties that are therapeutically useful. L-ficolin might contribute to viral clearance through activation of serine proteases activating the complement cascade. Complement is an essential component of the anti-viral immunity to other flaviviruses [39], and is likely to contribute to protection against HCV infection [22]. Indirect evidence supports an anti-viral role for the complement cascade, as both HCV NS5A and Core proteins inhibit complement component C4 transcription. Protectin (CD59) is also incorporated into HCV virions [40] suggesting that HCV has evolved multiple strategies to evade complement-dependent lysis. It is possible that L-ficolin mediated complement activation might contribute to *in vivo* elimination of HCV particles as well as infected cells.

We assayed the concentration of L-ficolin expressed in the serum of healthy individuals and people chronically infected with HCV, finding that the range is consistent with that previously reported in healthy cohorts [41]. While we appreciate that the localized concentration of L-ficolin might be higher at the site of synthesis in the liver, this provides evidence that neutralizing concentrations of L-ficolin are circulating in human sera. The proportion of HCV virions that circulate as complexes with L-ficolin remains to be determined. It was also noted that any liver damage caused by chronic HCV infection did not result in reduced L-ficolin expression in these individuals.

This study highlights the potential for an oligomeric recombinant L-ficolin as a novel therapeutic entry inhibitor for HCV infection. The broad, potent antiviral activity might also be applied to inhibition of entry of by other enveloped viruses possessing acetylated

glycoproteins. It also has identified a previously unreported antiviral activity of the L-ficolin ligand N-acetylcysteine.

Acknowledgments:

This work was funded by a studentship to MRH from the Egyptian government, EU FP7 (Health-F4-2012-305600), and the MRC DPFS (G0801169). Clinical samples were provided with ethical approval (MREC/98/3/55) by the Trent Clinical HCV Cohort. We thank Arvind Patel, Charles Rice Jens Bukh and Francois-Loic Cosset for kind provision of valuable reagents.

References

1 Freeman AJ, Dore GJ, Law MG, Thorpe M, Von Overbeck J, Lloyd AR, Marinos G, Kaldor JM: Estimating progression to cirrhosis in chronic hepatitis C virus infection. Hepatology 2001;34:809-816.

2 Londono MC, Crespo G, Forns X: Pretransplant and posttransplant treatment of hepatitis C virus infection with protease inhibitors. Curr Opin Organ Transplant 2013;18:271-278.

3 Edwards VC, Tarr AW, Urbanowicz RA, Ball JK: The role of neutralizing antibodies in hepatitis C virus infection. J Gen Virol 2012;93:1-19.

4 Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.

5 Bartosch B, Dubuisson J, Cosset FL: Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 2003;197:633-642.

6 Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM: Structural biology of hepatitis C virus. Hepatology 2004;39:5-19.

7 Iacob RE, Perdivara I, Przybylski M, Tomer KB: Mass spectrometric characterization of glycosylation of hepatitis C virus E2 envelope glycoprotein reveals extended microheterogeneity of N-glycans. J Am Soc Mass Spectrom 2008;19:428-444.

8 Goffard A, Callens N, Bartosch B, Wychowski C, Cosset FL, Montpellier C, Dubuisson J: Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. J Virol 2005;79:8400-8409.

9 Helle F, Vieyres G, Elkrief L, Popescu CI, Wychowski C, Descamps V, Castelain S, Roingeard P, Duverlie G, Dubuisson J: Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. J Virol 2010;84:11905-11915.

10 Brown KS, Keogh MJ, Owsianka AM, Adair R, Patel AH, Arnold JN, Ball JK, Sim RB, Tarr AW, Hickling TP: Specific interaction of hepatitis C virus glycoproteins with mannan binding lectin inhibits virus entry. Protein Cell 2010;1:664-674.
11 Helle F, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J: Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. J Biol Chem 2006;281:25177-25183.
12 Meuleman P, Albecka A, Belouzard S, Vercauteren K, Verhoye L, Wychowski C, Leroux-Roels G, Palmer KE, Dubuisson J: Griffithsin has antiviral activity against hepatitis C virus. Antimicrob Agents Chemother 2011;55:5159-5167.
13 Holmskov U, Thiel S, Jensenius JC: Collections and ficolins: humoral lectins of the innate immune defense. Annu Rev Immunol 2003;21:547-578.
14 Krarup A, Mitchell DA, Sim RB: Recognition of acetylated oligosaccharides by human L-ficolin. Immunol Lett 2008;118:152-156.
15 Matsushita M, Fujita T: Ficolins and the lectin complement pathway. Immunol Rev 2001;180:78-85.
16 Garlatti V, Belloy N, Martin L, Lacroix M, Matsushita M, Endo Y, Fujita T, Fontecilla-Camps JC, Arlaud GJ, Thielens NM, Gaboriaud C: Structural insights into the innate immune recognition specificities of L- and H-ficolins. EMBO J 2007;26:623-633.
17 Matsushita M, Endo Y, Taira S, Sato Y, Fujita T, Ichikawa N, Nakata M, Mizuochi T: A novel human serum lectin with collagen- and fibrinogen-like domains that functions as an opsonin. J Biol Chem 1996;271:2448-2454.
18 Le Y, Lee SH, Kon OL, Lu J: Human L-ficolin: plasma levels, sugar specificity, and assignment of its lectin activity to the fibrinogen-like (FBG) domain. FEBS Lett 1998;425:367-370.
19 Roozendaal R, Carroll MC: Complement receptors CD21 and CD35 in humoral immunity. Immunol Rev 2007;219:157-166.
20 Keirstead ND, Lee C, Yoo D, Brooks AS, Hayes MA: Porcine plasma ficolin binds and reduces infectivity of porcine reproductive and respiratory syndrome virus (PRRSV) in vitro. Antiviral Res 2008;77:28-38.
21 Pan Q, Chen H, Wang F, Jeza VT, Hou W, Zhao Y, Xiang T, Zhu Y, Endo Y, Fujita T, Zhang XL: L-ficolin binds to the glycoproteins hemagglutinin and neuraminidase and inhibits influenza A virus infection both in vitro and in vivo. J Innate Immun 2012;4:312-324.
22 Liu J, Ali MA, Shi Y, Zhao Y, Luo F, Yu J, Xiang T, Tang J, Li D, Hu Q, Ho W, Zhang X: Specifically binding of L-ficolin to N-glycans of HCV envelope glycoproteins E1 and E2 leads to complement activation. Cell Mol Immunol 2009;6:235-244.
23 Cseh S, Vera L, Matsushita M, Fujita T, Arlaud GJ, Thielens NM: Characterization of the interaction between L-ficolin/p35 and mannan-binding lectin-associated serine proteases-1 and -2. J Immunol 2002;169:5735-5743.
24 Michalak JP, Wychowski C, Choukhi A, Meunier JC, Ung S, Rice CM, Dubuisson J: Characterization of truncated forms of hepatitis C virus glycoproteins. J Gen Virol 1997;78 (Pt 9):2299-2306.
25 Yamada E, Montoya M, Schuettler CG, Hickling TP, Tarr AW, Vitelli A, Dubuisson J, Patel AH, Ball JK, Borrow P: Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets. J Gen Virol 2005;86:2507-2512.

26 Owsianka A, Tarr AW, Juttla VS, Lavillette D, Bartosch B, Cosset FL, Ball JK, Patel AH: Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J Virol 2005;79:11095-11104.
27 Tarr AW, Urbanowicz RA, Hamed MR, Albecka A, McClure CP, Brown RJ, Irving WL, Dubuisson J, Ball JK: Hepatitis C patient-derived glycoproteins exhibit marked differences in susceptibility to serum neutralizing antibodies: genetic subtype defines antigenic but not neutralization serotype. J Virol 2011;85:4246-4257.
28 Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D: Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J Virol 1998;72:2855-2864.
29 Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM: Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
30 Hummelshoj T, Thielens NM, Madsen HO, Arlaud GJ, Sim RB, Garred P: Molecular organization of human Ficolin-2. Mol Immunol 2007;44:401-411.
31 Yanagi M, Purcell RH, Emerson SU, Bukh J: Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc Natl Acad Sci U S A 1997;94:8738-8743.
32 Krarup A, Thiel S, Hansen A, Fujita T, Jensenius JC: L-ficolin is a pattern recognition molecule specific for acetyl groups. J Biol Chem 2004;279:47513-47519.
33 Matsushita M, Endo Y, Fujita T: Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. J Immunol 2000;164:2281-2284.
34 Duvet S, Cocquerel L, Pillez A, Cacan R, Verbert A, Moradpour D, Wychowski C, Dubuisson J: Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. J Biol Chem 1998;273:32088-32095.
35 Goffard A, Dubuisson J: Glycosylation of hepatitis C virus envelope proteins. Biochimie 2003;85:295-301.
36 Tarr AW, Lafaye P, Meredith L, Damier-Piolle L, Urbanowicz RA, Meola A, Jestin JL, Brown RJ, McKeating JA, Rey FA, Ball JK, Krey T: An alpaca nanobody inhibits hepatitis C virus entry and cell-to-cell transmission. Hepatology 2013;58:932-939.
37 Garlatti V, Martin L, Gout E, Reiser JB, Fujita T, Arlaud GJ, Thielens NM, Gaboriaud C: Structural basis for innate immune sensing by M-ficolin and its control by a pH-dependent conformational switch. J Biol Chem 2007;282:35814-35820.
38 Marzi A, Mitchell DA, Chaipan C, Fisch T, Doms RW, Carrington M, Desrosiers RC, Pohlmann S: Modulation of HIV and SIV neutralization sensitivity by DC-SIGN and mannose-binding lectin. Virology 2007;368:322-330.
39 Mehlhop E, Diamond MS: Protective immune responses against West Nile virus are primed by distinct complement activation pathways. J Exp Med 2006;203:1371-1381.
40 Amet T, Ghabril M, Chalasani N, Byrd D, Hu N, Grantham A, Liu Z, Qin X, He JJ, Yu Q: CD59 incorporation protects hepatitis C virus against complement-mediated destruction. Hepatology 2012;55:354-363.
41 Kilpatrick DC, Fujita T, Matsushita M: P35, an opsonic lectin of the ficolin family, in human blood from neonates, normal adults, and recurrent miscarriage patients. ImmunolLett 1999;67:109-112.

485

486

Figure Legends

Figure 1. Expression of recombinant, oligomeric human L-ficolin. A) Schematic illustrating the structure of L-ficolin. Each polypeptide monomer possesses a cysteine-rich N-terminal domain, a collagen-like domain, a neck region, and a C-terminal fibrinogen-like domain. Monomers oligomerize into dodecamers formed of trimeric subunits. B) L-ficolin was purified from human serum using GlcNAc-Sepharose. The flow-through (F), mannose wash (M), and L-ficolin eluate fractions (lanes 1-7) were detected by western blot with mAb GN5 following non-reducing (NR) or reducing (R) SDS-PAGE. Serum L-ficolin oligomers appeared as a 35kDa monomer, 70kD dimers and ~250kD oligomers, as well as higher-order multimers. C & D) Expression of recombinant L-ficolin. *In vitro* expressed L-ficolin was purified using an anti-FLAG M2 affinity resin. L-ficolin samples were resolved using non-reducing PAGE and western blotting with anti L-ficolin antibody, GN5 (C) or anti-FLAG mAb M2 (D). Each blot presents reactivity with start material (S), flow through (F) and eluted L-ficolin (E). Monomeric (35kDa), dimeric (~70kDa), and multimeric forms (≥250kD) of recombinant L-ficolin were observed. E) Eluted material was analyzed under non-reducing conditions (NR) or reduced in the presence of dithiothreitol (R), demonstrating the reduction of oligomers (o) to monomers (m).

Figure 2. Recombinant L-ficolin binds to HCV glycoproteins E1 and E2 in an acetyl-specific manner. A) L-ficolin binding to recombinant HCV glycoproteins was evaluated by ELISA. E2 was captured using mAb GN5-immobilised L-ficolin and detected by anti-

E2 antibody. Equivalent, dose-dependent interaction was observed between the soluble ectodomain of HCV E2 ($sE2_{661}$) and both recombinant L-ficolin (■) or purified serum L-ficolin (○). The highest concentration of $sE2_{661}$ was incubated with mAb GN5 in the absence of L-ficolin as a control (◆). B) Dose-dependent binding of L-ficolin to 293T cell lysates from cells transfected with E1/E2 (■), or mock-transfected cells (▲). C) Immobilized L-ficolin was pre-incubated with the indicated concentrations of L-ficolin ligands GlcNAc (▼),CysNAc (▲), or D-Mannose (■), prior to binding sE2. Binding is presented as a proportion of binding in the absence of inhibitor. IC_{50} values were 260mM (GlcNAc), and 1.8mM (CysNAc). No inhibition by D-Mannose was observed.

Figure 3. L-ficolin-mediated neutralization of HCV entry A) Retroviral pseudoparticles possessing envelope glycoproteins E1/E2 from strains of HCV representing genotypes 1a (H77c), 3a (UKN3A13.6), or 4a (UKN4.11.1), the VSV G protein, or HIV-1 gp160 were treated for one hour at room temperature with purified recombinant L-ficolin (grey bars) or a control fraction from purification containing no L-ficolin (black bars), before infecting Huh7 cells. All of the HCVpp and the VSV G pseudoparticles were neutralized by L-ficolin. No inhibition of entry of pseudoparticles bearing HIV-1 glycoproteins into TZM-bl cells was observed. Data is presented as the proportion of infectivity in the absence of inhibitor. B) FLAG-tagged L-ficolin expressed into the supernatants of HEK293T cells was fractionated by affinity purification on FLAG resin and pooled into three independent fractions (fractions F1, F2 and F3), as well as a negative fraction containing no L-ficolin (N) These fractions contained 157 μgmL^{-1}, 46 μgmL^{-1}, 10 μgmL^{-1} and 0 μgmL^{-1} total protein, respectively. C) When analyzed by staining an SDS-PAGE gel, protein was only detected in sample F1. D) The fractions were each diluted to 1 μgmL^{-1} total protein and

assessed for neutralization of entry of HCVpp bearing JFH1 E1/E2 glycoproteins. Data is presented as a proportion of an uninhibited control. Significant neutralization was only exhibited for the fraction containing detectable oligomeric L-ficolin (** $p<0.01$). E) The F1 fraction was found to neutralize entry of the JFH1 strain of cell-cultured HCV in a dose dependent manner (IC_{50}= 1.2µgmL^{-1}), while no inhibition was observed with the negative control sample.

Figure 4. Neutralization of HCV entry by L-ficolin requires interaction with virus particles. Protein fractions containing oligomeric L-ficolin, or a control with no detectable L-ficolin diluted equivalently, were incubated with either HCVpp (strain H77), or target Huh7 cells. Following washing, cells were infected with HCVpp. >50% neutralization was only observed when L-ficolin was pre-incubated with virus particles. A significant difference was observed between virus and cells treated with L-ficolin, compared to cells treated alone (1-way ANOVA; * $p<0.05$). No significant differences in entry were observed when cells were treated with L-ficolin or a negative control.

Figure 5. Inhibition of HCV entry is calcium-dependent. A) Infection assays were prepared with cell-culture grown HCV. Neutralization was performed with approximately 50% neutralizing concentrations of oligomeric L-ficolin in the presence of 2mM $CaCl_2$ (black bars) or 7mM $CaCl_2$ (grey bars). Neutralization was compared to a positive uninhibited control, and a sample treated with an equivalent L-ficolin-negative fraction from purification. Greater neutralization of HCVcc was observed with L-ficolin fractions in the presence of 7mM $CaCl_2$. (One-way ANOVA; ** $p<0.01$, *** $p<0.001$). B) Ficolin was

incubated in PBS +/-7mM $CaCl_2$ before analysis by SDS-PAGE/western blotting with mAb GN5. No difference in oligomerization was observed in the presence of $CaCl_2$.

Figure 1.

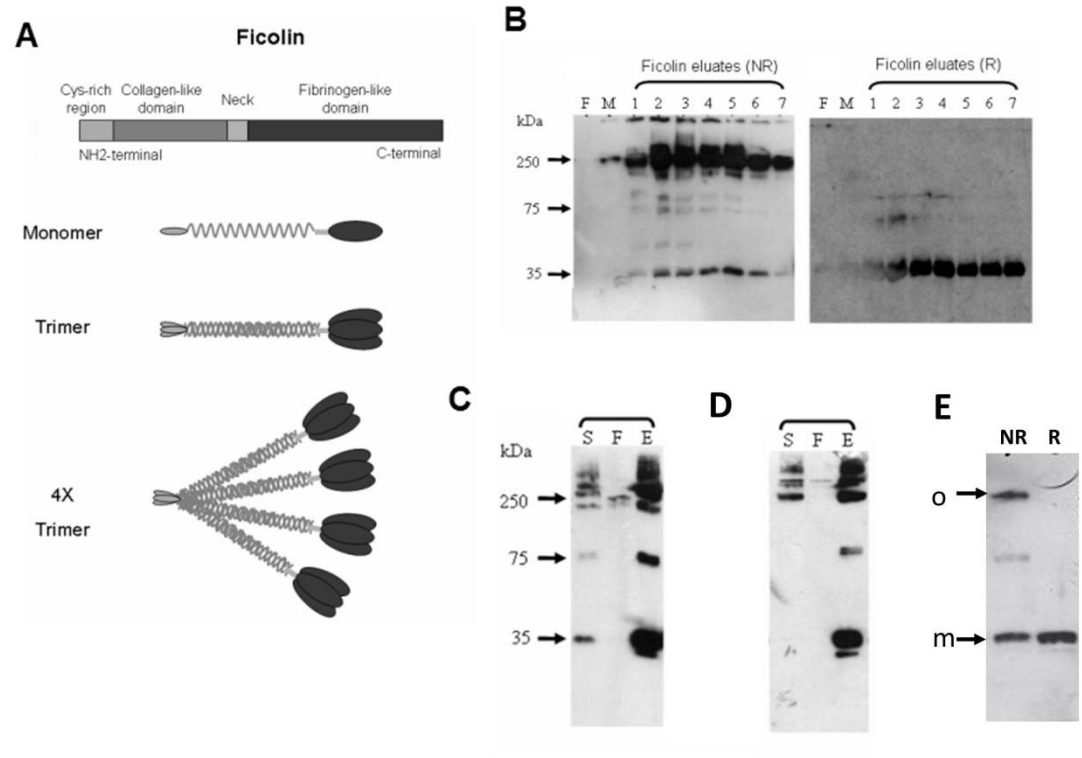
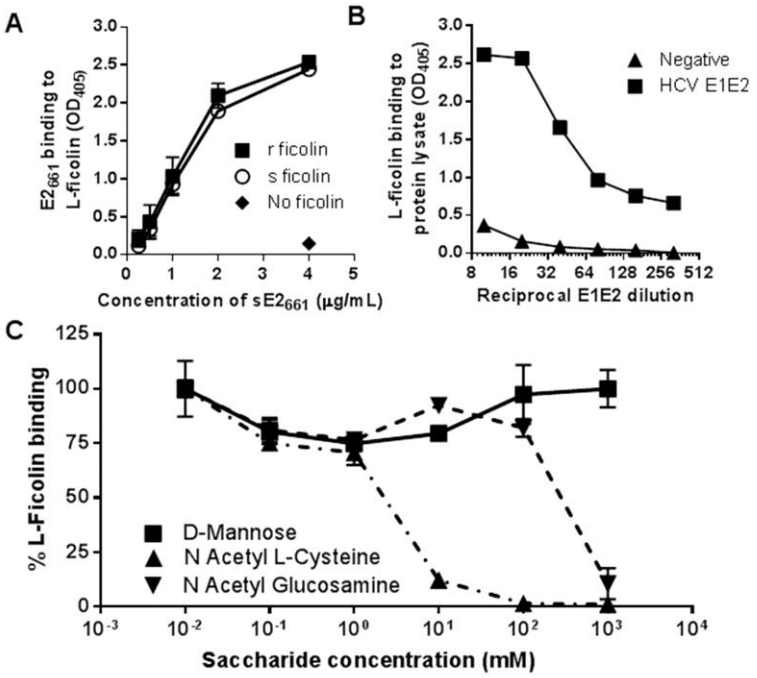
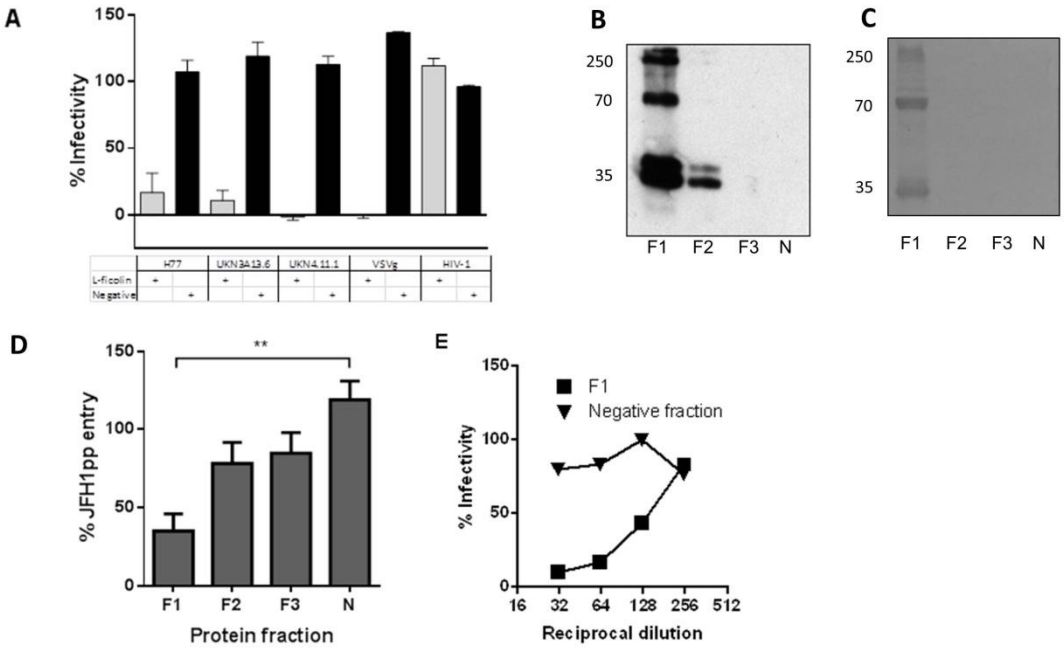


Figure 2.


A
B
C
r ficolin
s ficolin
No ficolin
Concentration of sE2661 (µg/mL)
Negative
HCV E1E2
Reciprocal E1E2 dilution
% L-Ficolin binding
D-Mannose
N Acetyl L-Cysteine
N Acetyl Glucosamine
Saccharide concentration (mM)

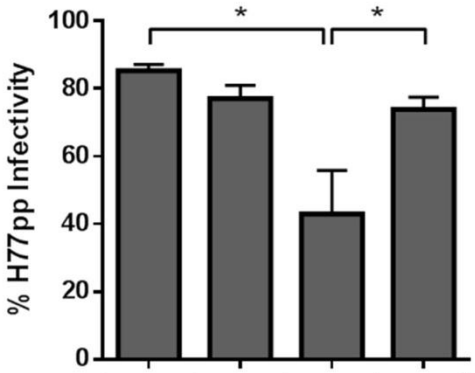
562

Figure 3.


A
% Infectivity
150
100
50
0
H77
UKN3A13.6
UKN4.11.1
VSVg
HIV-1
L-ficolin
Negative
B
250
70
35
F1 F2 F3 N
C
250
70
35
F1 F2 F3 N
D
% JFH1pp entry
**
F1 F2 F3 N
Protein fraction
E
% Infectivity
F1
Negative fraction
16 32 64 128 256 512
Reciprocal dilution

563

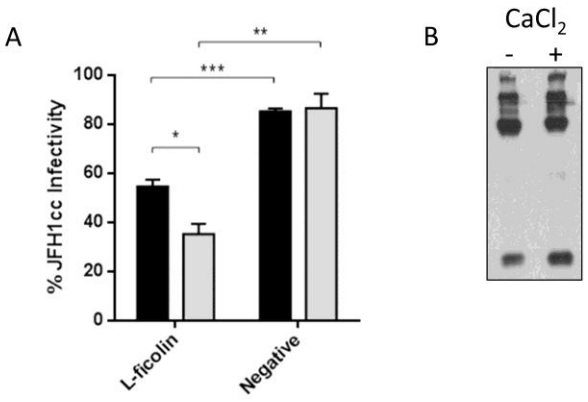
Figure 4.



Cells preincubated with				
Oligomeric L-ficolin	+			
Negative fraction		+		
HCVpp preincubated with				
Oligomeric L-ficolin			+	
Negative fraction				+

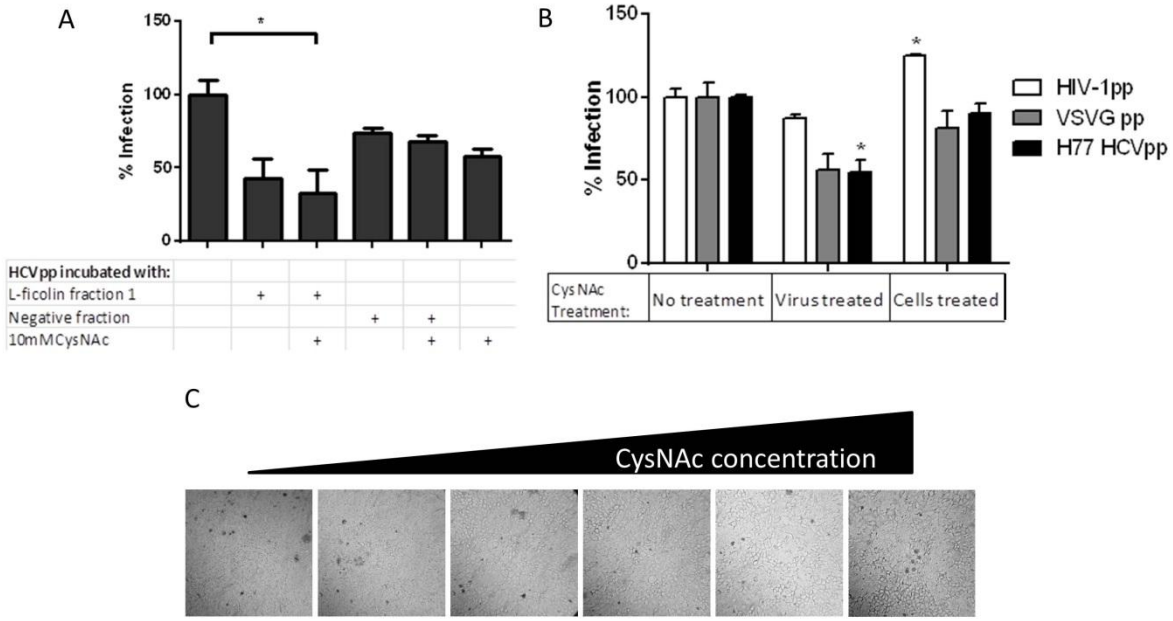
564

Figure 5.


A
B
CaCl2
-
+
**
***
*
100
80
60
40
20
0
% JFH1cc Infectivity
L-ficolin
Negative

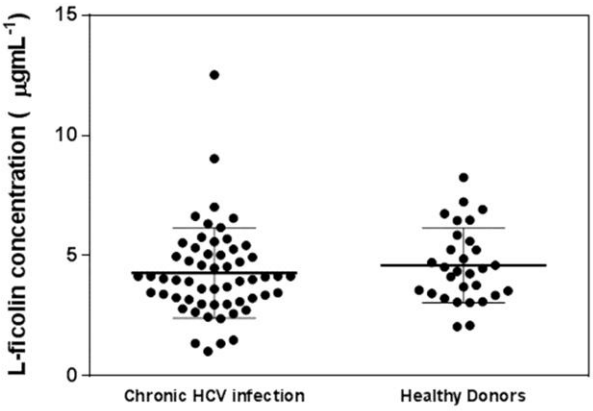
565

Supplementary Figure 1.



566

Supplementary Figure 2.



567