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Glucocorticosteroids increase cell entry by hepatitis C virus

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Abbreviations

Hepatitis C Virus (HCV), Cyclosporin A (CsA), Scavenger receptor class B type I (SR-BI), quantitative reverse transcriptase PCR (qRT-PCR), glucocorticosteroids (GCs)

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Abstract

Background & Aims: Corticosteroids are used as immunopressants in patients with autoimmune disorders and transplant recipients. However, these drugs worsen hepatitis C virus (HCV) recurrence after liver transplantation, suggesting that they may directly exacerbate HCV infection. **_Methods:_** The influence of immunosuppressive drugs on HCV replication, assembly and entry was assessed in Huh-7.5 cells and primary human hepatocytes using cell culture- and patient-derived HCV. Replication was quantified by immunofluorescence, luciferase assays, quantitative reverse transcriptase PCR, or core ELISAs. Expression of HCV entry factors was evaluated by cell sorting and immunoblot analyses. **_Results:_** Glucocorticosteroids slightly reduced HCV RNA-replication, but increased efficiency of HCV entry by up to 10-fold. This was independent of HCV genotype but specific to HCV, because vesicular stomatitis virus glycoprotein-dependent infection was not affected by these drugs. The increase in HCV entry was accompanied by upregulation of mRNA and protein levels of occludin and the scavenger receptor class B type I (SR-BI) - 2 host cell proteins required for HCV infection; increase of entry by glucocorticosteroids was ablated by RU-486, an inhibitor of glucocorticosteroid signaling. Glucocorticosteroids increased propagation of cell culture-derived HCV ca. 5 to 10-fold in partially differentiated human hepatoma cells, and increased infection of primary human hepatocytes by cell culture- and patient-derived HCV. **_Conclusions:_** Glucocorticosteroides specifically increase HCV entry by upregulating the cell entry factors occluding and SR-BI. Our data suggest that the potential effects of high dose glucocorticosteroids on HCV infection in vivo may be due to increased HCV dissemination.

Key words

Hepatitis C virus, glucocorticosteroids, immunosuppressive therapy, virus entry, scavenger receptor class B type I, occludin

Introduction

More than 130 million people are chronically infected with HCV. End stage liver disease associated with chronic HCV infection is a leading indication for liver transplantation [1]. However, reinfection is universal and more than 25 % of patients develop recurrent hepatitis in the donor organ.

HCV infects hepatocytes and establishes a chronic infection in the majority of cases. Viral isolates are grouped into six genotypes and more than 100 subtypes [2]. Chronic HCV infection is treated with pegylated Interferon alpha (PEG-IFN-α) and ribavirin curing 80% of genotype 2 and 3 and approximately 50% of genotype 1-infected individuals [1]. PEG-IFNα and ribavirin have also been applied in patients after liver transplantation. However, in this context, efficacy is lower with sustained virological responses ranging from 20% to 45% only [3].

Notably, the outcome of hepatitis C after liver transplantation has worsened during the last 10-15 years [4]. Several factors have been suspected to be accountable including an increased donor age [5]. Generally, HCV RNA levels are higher after transplantation than before and high virus titers are associated with a worse long term outcome [6], [7]. The immunosuppressive treatment has been supposed to alter the course of hepatitis C in liver transplant recipients. While cyclosporin A (CsA) and mycophenolate mofetil (MMF) have antiviral effects in tissue culture and m-TOR antagonists have antifibrotic properties in vivo [8-10], use of corticosteroids has been a matter of debate. Repeated usage of high doses of corticosteroids have been associated with more rapid fibrosis progression and poor long-term outcome of graft hepatitis C [11-12]. Nevertheless, steroid boli therapies are still applied after liver transplantation for hepatitis C, and the molecular mechanisms responsible for the steroid-treatment associated exacerbation of HCV infection are currently unclear.

Recently, a cell culture system permissive for HCV replication and reproducing the complete replication cycle was developed [13]. This HCV infection model is based on the JFH1 strain (genotype 2a), which replicates efficiently in Huh-7 cells. These advances allowed us to

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assess the direct impact of immunosuppressive agents on HCV RNA replication, virus production and infectivity.

Material and Methods

Drugs:

Cyclosporin A, everolimus, basiliximab, and enteric-coated mycophenolate sodium were provided by Novartis, Basel, Switzerland. Tacrolimus, azathioprine, dexamethasone, prednisolone, fludrocortisone, cholesterol, and RU-486 were purchased from Sigma-Aldrich, Seelze, Germany or from Sanofi Aventis, Frankfurt, Germany.

Plasmids:

The reporter virus genome Luc-Jc1 and expression plasmids for HCV E1/E2 proteins of the J6CF (genotype 2a) or the Con1 isolate (genotype 1b), have been described recently [14-15, 16]. The murine leukemia virus (MLV) based retroviral vector pRV-F-Luc-IZ is a derivative of pczCFG5-IZ [17]. Briefly, this vector carries a chimeric 5´LTR in which the MLV-U3 region was replaced by a cytomegalovirus immediate early promoter. The transgene (firefly luciferase) is inserted into the env locus of the original MLV genome and is followed by the internal ribosome entry site of the encephalomyocarditis virus (EMCV) and the *ble* resistance gene conferring resistance to Zeozin[TM] (Invitrogen, Karlsruhe, Germany).

Cell Culture:

Low passage Huh-7.5 cells were cultured in Dulbecco`s modified Eagle medium (DMEM, Invitrogen) with 10% fetal bovine serum (FCS Gold, PPA, Coelbe, Germany) , 1x non-essential amino acids (Invitrogen), 100µg/ml streptomycin (Invitrogen) and 100 IU/ml penicillin (Invitrogen).

HCV luciferase replication and infection assays

Huh-7.5 cells were transfected and infected as described recently [14]. Drugs were applied as indicated in the text.

Preparation of retroviral pseudo-particles

MLV-based pseudotypes were generated by transfection of 293T cells. Briefly, 1.4×10^6 293T cells were seeded in 6-cm diameter plates 1 day before transfection with 2.7 µg of envelope protein expression construct (pczVSV-G [20] or pcDNA3ΔcE1E2-J6CF or pcDNA3ΔcE1E2-Con1 [16]) and 2.7 µg of a firefly luciferase transducing retroviral vector (pRV-F-Luc-IZ) and 2.7 µg of MLV gag/pol expression plasmid pHIT60 [21] by using Lipofectamine 2000 (Invitrogen). Pseudo-particles were harvested 48 h later, cleared by passage through 0.45-µm-pore-size filters, and used for infection assays. Luciferase activity in the infected cells was measured 48 h after inoculation as described recently [14].

Quantitative detection of HCV RNA and core protein.

HCV core protein was measured using an HCV core antigen kit (Wako Chemicals, Neuss, Germany). RNA was isolated from infected cells using a Nucleo Spin RNAII Kit (Macherey-Nagel, Düren, Germany). HCV RNA was quantified using a Light Cycler 480 (Roche, Mannheim, Germany) as described recently[19].

Flow cytometry

Huh-7.5 cells were stained with SR-BI- (Novus Biologicals Littleton, CA) or CD81-specific antibodies (clone JS-81, Becton Dickinson, Heidelberg, Germany) at 4°C in phosphate buffered saline (PBS) supplemented with 2% FCS for 20 min. The unpermeabilized cells were washed twice and immediately analysed for surface expression on a fluorescence-activated cell sorter (FACScalibur, Becton-Dickinson). Data were analysed using FloJo software (Tree Star, Inc. Oregon Corporation, Ashland, OR).

Western blot analysis

Cells were washed once with PBS and lysed in sample buffer (400 mM Tris, pH 8.8, 10 mM EDTA, 0.2% bromophenol blue, 20% sucrose, 3% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol). Proteins resolved by electrophoresis and blotted onto a polyvinylidene difluoride membrane were detected using claudin-1 (anti mouse, clone 2H10010, Zymed,

Invitrogen), SR-BI (anti rabbit, Novus Biologicals Littleton, CA) or occludin-specific antibodies (anti mouse, clone OC-3F10, Zymed, Invitrogen) at a dilution of 1:500, and the ECL Plus Western Blotting Detection System (GE Healthcare Europe, Freiburg, Germany). Signals were quantified using Intas Lab Image 1D software (Intas Science imaging Instruments, Göttingen, Germany) and normalized for the actin-specific signal.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The mRNA levels of SR-BI, occludin and GAPDH were quantified using the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany), the QuantiTect Primer Assay kit (Qiagen) and gene-specific primers.

Indirect immunofluorescence

Huh-7.5 cells were seeded onto glass coverslips in 24-well plates at a density of 2×10^4 cells/cm^2 per well and were fixed 48 h post infection using PBS supplemented with 3% paraformaldehyde for 10 min at room temperature. Staining of NS5A was performed as described [19] using the 9E10 hybridoma supernatant at a dilution of 1:2,000.

Immunohistochemical staining and virus titration

Virus titers were measured using the limiting dilution assay as described recently [19]. Virus titers (50% tissue culture infective dose [TCID$_{50}$/ml]) were calculated based on the method of Spearman and Kärber [22-23].

Human Primary Hepatocyte Cultures

We obtained liver tissue from donors undergoing hemihepatectomy or atypic liver resection without primary liver disease (e.g. colorectal metastases or metastases of a mamma carcinoma or melanoma). These donors were negative for Hepatitis A, B and C and HIV and gave their written consent for this study.

Hepatocytes were isolated from an encapsulated liver sample by a two-step perfusion technique [24]. Cells were inoculated with HCVcc or patient sera (genotype 1; viral load >

5×10^6 IU/ml; HAV, HBV, HIV negative) for 3h. HCV replication was measured by qRT-PCR up to two weeks after infection.

The study was approved by ethics committee of the Hannover Medical School. Patients provided written informed consent.

Results

Glucocorticosteroids (GCs) slightly reduce HCV RNA replication but increase infectivity

We evaluated the effects of common immunosuppressive drugs on HCV replication and infectivity, using firefly luciferase reporter viruses based on the intragenotypic genotype 2a chimera Jc1 (Luc-Jc1; Fig. 1A [14]). These results are summarized in Table I. In agreement with previous data [25], a high dose of the GC prednisolone decreased replication of Luc-Jc1 in transfected Huh-7.5 cells by ca. 50% (Fig. 1B). However, in the complete life cycle assay we noted a moderate and dose dependent stimulation of HCV propagation (Fig. 1C). To determine if this is due to differential activity (inhibitory/stimulatory) of the drug on distinct phases of the viral replication cycle, we assessed the efficiency of virus release in the presence of prednisolone. The amount of extracellular core protein, a structural component of HCV virions, was not markedly altered by the drug (Fig. 1D). However, when viruses prepared in the absence of prednisolone were used to infect naïve Huh-7.5 cells and the drug was added during inoculation and until harvesting, we observed a dose-dependent stimulation of infection (Fig. 1E). These data provide strong evidence that this corticosteroid enhances the efficiency of HCV entry.

To investigate if this effect is specific for corticosteroids with GC activity, we compared the influence of dexamethasone, (a synthetic GC with no relevant mineralcorticoid activity), the synthetic fludrocortisone which primarily has mineralcorticoid activity, and cholesterol on HCV infection. Among these, only dexamethasone stimulated HCV infectivity arguing that the effect on HCV is specific to GCs (Fig. 1F).

Notably, unlike during sustained steroid maintenance therapy standard bolus treatment for acute rejection involves high doses of methylprednisolone (500 mg/day for three consecutive days) which result in GC plasma levels of ca. 100µg/ml [26] [27] Thus, our data suggest that GC doses similar to those for in vivo bolus therapy slightly reduce HCV RNA replication but strongly increase virus infectivity with the latter effect dominating over the former in the context of a complete replication cycle.

HCV entry is enhanced by GCs

To characterize the stimulation of virus entry by GCs, we administered prednisolone at different intervals during the early phase of infection. As shown in Figure 2A, application of prednisolone shortly before, during, or directly after inoculation increased HCV infectivity, while thereafter infection was not changed. Notably, addition of the drug shortly prior to the virus stimulated infection, suggesting that GCs transiently act on the cell surface rather than the virion.

To specifically assess the influence on HCV entry, we utilized HCV pseudoparticles (HCVpp). Since these are retroviral cores carrying HCV glycoproteins in their envelope [28-29], only the early steps of virus entry are HCV dependent, i.e. virus binding, uptake and virus-membrane fusion, whereas all later steps are dependent on retroviral proteins. Using this approach we observed that stimulation by prednisolone is HCV specific, since retroviral pseudotypes with the glycoprotein of the vesicular stomatitis virus (VSV-G) were not affected (Fig 2B). As infectivity of HCVpp with J6CF (genotype 2a) or Con1 (genotype 1b) glycoproteins was stimulated, the enhancement of HCV infection by prednisolone is not genotype- or isolate-specific (Fig. 2B).

To analyze the mechanism by which GCs facilitate infection, we investigated the kinetic of HCV uptake into Huh-7.5 cells. To this end, we employed two potent inhibitors of HCV entry, namely CD81-specific antibodies which block interaction of HCV with this crucial entry factor, and concanamycin A, an inhibitor of endosomal acidification, which prevents the low pH-dependent viral fusion mechanism. These agents were either added together with the virus (Fig. 2C time point 0 min) or with a delay of varying duration to find out when the virus is resistant to the respective compound.

Interestingly, HCV was resistant to CD81-specific antibodies and inhibitors of low pH-induced fusion (Con A) more rapidly in the presence of prednisolone compared to the solvent control (Fig. 2C). Control antibodies (CD13) had no effect irrespective of prednisolone and time point of application. These results imply that GCs accelerate both uptake of HCV into host cells

(acquisition of resistance to CD81 antibodies) and escape from endosomes (concanamycin A resistance) indicating that GCs facilitate infection by enhancing the kinetic of (an) early step(s) of HCV entry.

The GC prednisolone upregulates SR-BI cell surface expression and occludin expression

Due to these results we hypothesized that GCs modulate the abundance of host factors crucial for virus entry, and investigated expression of proteins implicated in HCV infection. We analyzed expression of all four key entry factors of HCV, i.e. CD81 [30], SR-BI [31], claudin-1 [32], and occludin [33]. Since HCV is associated with lipoproteins of low and very low density [34-35], natural ligands of the LDL receptor, and since LDL-R was reported to function during HCV entry [36], we also determined LDL-R expression. As shown in Figure 3A, claudin-1 and LDL-receptor expression were not affected by prednisolone. However, occludin was upregulated by prednisolone to levels ca. 18-fold higher compared to untreated cells (Fig. 3A).

While CD81 surface expression was not altered, the abundance of SR-BI at the cell surface increased 3-fold (Figure 3B). These results indicate that GCs upregulate expression of two key host factors necessary for productive HCV infection. Interestingly, the upregulation was rapid and transient (Supplementary Fig. 1) which is in agreement with the kinetics of the stimulatory effect of GCs on HCV cell entry.

RU-486, an antagonist of GC signalling, counteracts upregulation of SR-BI and occludin mRNA and protein level and also enhancement of HCV entry

To analyze how prednisolone upregulates SR-BI and occludin expression, we quantified mRNA levels of both proteins in prednisolone-treated Huh-7.5 cells using qRT-PCR. These analyses revealed that prednisolone treatment for 5 h increased the abundance of both mRNAs in a dose-dependent manner, while copy numbers of GAPDH were unaffected (Fig. 4A). To establish a direct link between GC signalling, upregulation of mRNA and protein levels of SR-BI and occludin, and enhancement of HCV entry, we utilized RU-486, a drug

that antagonizes GC signalling by direct binding to the GC receptor [37]. Remarkably, 10 µg/ml RU-486 fully abolished stimulation of HCV cell entry by prednisolone (Fig. 4B) and at the same time interfered with prednisolone-dependent accumulation of SR-BI and occludin mRNA and protein levels (Fig 4C and D). Notably, RU-486 treatment alone had no effect on HCV infectivity and replication (Supplementary Fig. 2). Direct knock down of SR-BI and occludin mRNA by siRNA interference showed inhibitory effects, but the results were inconclusive due to inhibitory effects also seen for control scrambled RNA (data not shown).. Together, these results agree with previous findings implicating GCs with upregulation of SR-BI and occludin [38-40] and provide strong evidence that prednisolone enhances HCV entry via GC signalling-dependent upregulation of these HCV entry factors.

Enhancement HCV infection and dissemination by GCs is cell density-dependent

Since claudin is a component of tight junctions and as these structures likely form in a cell density-dependent manner, we assessed the influence of cell density on the degree of infection enhancement by prednisolone. To this end, Huh-7.5 cells were seeded in different cell densities and infected with Luc-Jc1 in the presence or absence of prednisolone. Remarkably, the stimulatory effect of prednisolone was completely lost at low cell density, first visible at 1.5×10^4 cells/cm^2 and steadily increased up to ca. 10 fold at 2×10^5 cells/ cm^2 (Figure 5A).

To investigate whether GCs facilitate entry and as a consequence also dissemination of wild type HCV in tissue culture, we utilized the Jc1 chimera. When inoculating Huh-7.5 target cells with a low dose of Jc1 in the presence or absence of the drug, as early as 48 h post inoculation we noted an increase in the number of virus infected cells in the presence of prednisolone (Fig. 5B) suggesting that GCs enhance virus dissemination.

Prednisolone enhances chronic infection of differentiated Huh-7.5 cells

To mimic conditions more closely related to an in vivo infection of quiescent liver tissue-resident hepatocytes, we took advantage of Huh-7.5 cells partially differentiated by DMSO.

Sainz et al. recently reported that application of DMSO caused a growth-arrest and highly differentiated state of Huh-7.5 cells which then could be chronically infected with HCVcc for weeks [41]. After treating Huh-7.5 cells for 14 days with DMSO, confluent cultures were infected with Jc1 and permanently treated with different amounts of prednisolone. Cell culture supernatants were collected at various time points and analyzed for virus infectivity and HCV RNA. Interestingly, prednisolone doses of either 50 µg/ml or 200 µg/ml yielded substantially higher infectious titers throughout the complete follow up (Fig. 6A). Similarly, both, HCV Core amounts (data not shown) and HCV RNA (Fig. 6A, right panel) in the culture fluid were increased in the chronically infected cultures in the presence of prednisolone.

Prednisolone enhances infection of primary human hepatocytes by cell culture- and patient-derived HCV

Although human hepatocellular carcinoma derived cell lines sustain the complete HCV replication cycle, these cells functionally differ from primary human hepatocytes. Therefore, primary human hepatocytes were inoculated in the presence or absence of prednisolone either with a Jc1 or patient-derived HCV. To distinguish input RNA of the inoculum from de novo RNA replication, we performed control infections of the same batch of hepatocytes in the presence of CsA and pegylated IFN-α. As shown in Figure 5, both Jc1 as well as a representative patient serum infected the primary human hepatocytes as evidenced by 10- to 100-fold and almost 1,000-fold higher HCV RNA copy numbers compared to CsA treated controls, respectively (Fig. 6B and C). When adding 100 µg/ml prednisolone, Jc1 RNA levels were slightly elevated throughout the time course. Importantly, HCV RNA copy numbers in case of the patient serum were about 5-fold higher when prednisolone was present. In line with our data described above, increased HCV propagation in PHHs was also accompanied by upregulation of SR-BI and occludin mRNAs (Supplementary Fig. 3).These data indicate that GCs not only enhance infection of hepatoma cell lines by cell culture-derived HCV particles but also facilitate propagation of these viruses as well as of natural HCV from patients in primary human hepatocytes.

Discussion

In this study we observed that GCs slightly reduce HCV RNA replication, while strongly enhancing HCV infectivity. This stimulatory effect was associated with an upregulation of expression of two essential HCV entry factors (occludin and SR-BI) via signalling through the GC receptor. These results highlight a novel mechanism how GCs may also increase HCV infection in vivo.

Studying the effects of corticosteroids on HCV Con1 subgenomic replicon cells, Henry et al noted slightly reduced HCV RNA replication [25]. These authors therefore assumed that adverse effects of corticosteroids on HCV-infection were due to dampening of immune responses and thus indirect. While this is likely one mechanism that facilitates HCV replication in vivo, our findings suggest that GCs directly increase HCV dissemination through enhancement of HCV entry. The steroid-induced relative increase in HCV infectivity was more pronounced than the reduction in HCV replication. Importantly, this was not only true in reporter assays that test either entry or RNA replication. Also when using reporter viruses and an assay for the complete replication cycle, we noted a significant increase in virus propagation due to GCs. Moreover, HCV spread more rapidly in naive and in DMSO treated differentiated Huh-7.5 cells in the presence of prednisolone. Together these data indicated that the enhancement of virus entry was dominant over the minor inhibition of RNA replication.

Analyzing the mechanism permitting increased infection and more rapid virus spread, we made two important observations: First, we noted faster virus uptake and escape from endosomes in the presence of prednisolone. As a consequence, susceptibility of HCV infection to blockade by CD81-specific antibodies or concanamycin A, was lost more quickly. In vivo accelerated virus entry may contribute to immune evasion via escape from virus-neutralizing antibodies. While this kinetic effect may partially explain a more rapid spread in tissue culture, we believe that the latter is primarily due to an overall enhancement of virus infection. This direct stimulation of HCV infection may be most directly evident in our experiments with retroviral pseudotypes. Since these particles consist of retroviral cores

enveloped by HCV glycoproteins E1, E2, they specifically permit assessment of utilization of host cell entry factors by HCV. Importantly, while pseudotypes with E1-E2 complexes from two different isolates were stimulated, those particles carrying VSV-G were not. These data imply that steroid-dependent facilitation of uptake and infection does not universally apply to enveloped viruses, may be general for HCV, but at least does not only affect a single HCV isolate.

Our second finding relates to the mode by which GCs specifically enhance HCV infection: Our results indicate that this is due to upregulation of SR-BI and/or occludin, two host factors crucial for HCV infection. The increase of mRNA and protein levels and also enhancement of HCV infection were ablated by pre-treatment with RU-486, an antagonist of GC signalling. Moreover, fludrocortisone and cholesterol did not stimulate HCV infection. Therefore, we can rule out that interactions of GCs or more generally of compounds that share a steroid scaffold are sufficient to increase HCV infection in a rather non-specific manner. Rather, we conclude that GCs increase abundance of SR-BI and occludin via signalling through the GC receptor thus stimulating HCV cell entry.

 It is puzzling that at low cell density GCs did not enhance HCV infection. However, it is possible that under these conditions neither entry factor limits HCV infection. In fact, the cell density-dependence of GCs to stimulate HCV infection may be a reflection of HCVs utilization of tight junction proteins for cell entry. It is likely that formation of tight junctions in Huh7.5 is cell density-dependent and it is possible that incorporation o HCV entry factors (claudin-1 and occludin) into these complexes may alter their structure/function and as a consequence their utility for HCV infection. For instance, occludin may be poorly accessible in this context thus impeding HCV infection. Therefore, upregulation of occludin abundance may strongly facilitate infection particularly in the setting of high cell confluency. Further studies addressing the cell density-dependent abundance and subcellular localization of HCV entry factors will be important to resolve this question. Hepatocytes in the environment of the liver possess tight junction. Therefore, our experiments under high cell density conditions may best mimic the conditions in vivo.

When assessing HCV long term propagation in DMSO-treated differentiated Huh-7.5 cells we confirmed that prednisolone facilitates HCV propagation. Similarly, infection of primary human hepatocytes by cell culture- and patient-derived HCV was more efficient in the presence of the drug. Taken together, these data provide firm proof that high doses of GCs facilitate HCV infection in vitro. Based on these observations we propose that steroid bolus treatment (> 250 mg prednisolone/day) of HCV infected individuals which results in plasma levels up to 50µg/ml may foster virus dissemination through facilitation of virus entry into hepatocytes thus aggravating HCV recurrence. These data may have important implications for the understanding why repeated usage of high doses of corticosteroids has been associated with more rapid fibrosis progression and poor long-term outcome of graft hepatitis C.

Acknowledgement

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Table 1: Influence of immunosuppressive drugs on HCV replication and infection.

Compound	Mode of action	IC$_{50}$ (Replication)[a]	IC$_{50}$ (Infectivity)[a]	TC$_{50}$ (Toxicity)[b]
Cyclosporin A	Calcineurin inhibitor via cyclophilins	0.15 µg/ml	0.05 µg/ml	44 µg/ml
Tacrolimus (FK506)	Calcineurin inhibitor via FKBP12	NA[c]	NA[c]	8 µg/ml
Mycophenolate Sodium (MPA)	Inhibition of IMP dehydrogenase	23 µg/ml	15 µg/ml	120 µg/ml
Basiliximab	IL2 receptor antibody	NA[c]	NA[c]	8 µg/ml
Everolimus	m-Tor antagonist	NA[c]	2 µg/ml	5 µg/ml
Prednisolone	Inhibition of IL2 promotor	50 µg/ml	NA[c]	290 µg/ml
Azathioprine	Purine synthesis inhibitor	22 µg/ml	18 µg/ml	220 µg/ml

[a] Replication and infection assays were conducted as described in Figure 1

[b] Cytotoxicity was estimated using a standard cytotoxicity assay according to the instructions of the manufacturer (Promega)

[c] Not applicable

Figure legends

Figure 1: Differential effect of prednisolone on HCV RNA replication and infection. (A) Schematic representation of Jc1 and Luc-Jc1 constructs. (B) Huh-7.5 cells were transfected with Jc1-Luc, and prednisolone was added 4 h later. HCV replication was determined 48 h post transfection using luciferase assays and is expressed as % of cells treated with solvent alone. (C) Culture fluid of these cells was used to inoculate naïve Huh-7.5 cells. After 4 h, fresh medium with or without prednisolone was supplied. Mean values and the standard deviation of four independent experiments are shown. (D) Huh-7.5 cells were transfected with Luc-Jc1 and treated with prednisolone. 48 h later, intracellular and extracellar levels of core were determined. Mean values and the standard deviation of two independent experiments are shown. (E) Huh-7.5 cells were inoculated with Luc-Jc1 prepared in the absence of drugs. During inoculation and until measurement 48 later, prednisolone was added. Results are depicted as fold increase of HCV infectivity compared to cells treated with solvent. Mean values including the standard deviation of three independent experiments are shown. (F) Infection experiments as described in part (E) were performed using dexamethasone, fludrocortisone, and cholesterol.

Figure 2: Prednisolone facilitates HCV infection and increases kinetics of virus uptake. (A) Huh-7.5 cells were inoculated for 4 h (0h -4h) with Luc-Jc1 virus particles prepared in the absence of prednisolone. The drug was added at the indicated intervals. Mean values of two independent experiments including the standard deviation are shown. (B) MLV-based HCV pseudotypes with J6- or Con1-derived glycoproteins were used to infect Huh-7.5 cells in the presence or absence of prednisolone. MLV-pseudotypes with glycoproteins of the vesicular stomatitis virus (VSV-G) or without glycoproteins (pcDNA) served as control. Mean values of quadruplicate measurement including the standard deviation are given. (C) Kinetics of HCV infection in the presence of prednisolone was assessed by addition of CD81-specific antibodies (α-CD81), or concanamycin A (ConA), at various time points during the early

phase of infection. Antibodies to CD13 served as control. Infections were performed either in the absence (open squares) or the presence of 100 µg/ml prednisolone (filled squares). In each case Luc-Jc1 particles were adsorbed to Huh-7.5 cells at 4°C to synchronize infection. The infection efficiency of Luc-Jc1 in the presence of solvent was set to 100%. Mean values of two independent experiments including the standard deviation are shown.

Figure 3: Prednisolone-dependent expression of host factors crucial for HCV infection.

Expression of claudin-1, occludin and LDL-R in the presence or absence of prednisolone was determined by western blot (A) or for SR-BI and CD81 by FACS analysis (B).

Figure 4: Prednisolone-dependent upregulation of mRNA and protein level of occludin and SR-BI and enhancement of HCV infection is counteracted by RU-486

Occludin, SR-BI and GAPDH mRNA abundance was measured by qRT-PCR after treating cells for 5 h with increasing amounts of prednisolone (A), orafter pretreatment with RU-486 (B). (C) Protein expression of occludin and SR-BI were measured after pre-treatment with RU-486 in the presence or absence of prednisolone. (D) Huh-7.5 cells were preincubated with different doses of RU-486 for 30 minutes, washed and inoculated with Luc-Jc1 prepared in the absence of prednisolone. During inoculation, prednisolone was added at the indicated concentrations. Mean values of two independent experiments including the standard deviation are shown.

Figure 5: Prednisolone enhances HCV infection and dissemination in a cell density-dependent fashion

(A) Huh-7.5 cells were seeded at different densities ranging from $5 \times 10^3/cm^2$ to $2 \times 10^4/cm^2$ and were inoculated in the presence or absence of prednisolone. Infection efficiency was determined 48 h later by luciferase assays. Mean values of quadruplicates including the standard deviation are given. (B) Cells were inoculated with a low dose of Jc1 in the presence or absence of the drug and were fixed 48 h later. Indirect immunofluorescence was

conducted using NS5A-specific monoclonal antibodies. The number of infected cells was quantified by counting cells in three individual microscopic pictures that were arbitrarily chosen. Mean values including the standard deviation are given.

Figure 6: Prednisolone facilitates propagation of HCV in DMSO-treated differentiated Huh-7.5 cells and primary human hepatocytes. (A) Huh-7.5 cells were differentiated with DMSO as described [41]. After 14 days these cells were infected with Jc1 and permanently treated with different amounts of prednisolone. Cell culture supernatants were collected and were titrated on Huh-7.5 cells (left panel). HCV RNA copies in the culture fluid of the inoculated cells were monitored by qRT-PCR. Mean values of duplicate measurements are given (right panel). (B, C) Primary human hepatocytes were inoculated with either Jc1 (B) or a patient serum (C) in the presence of indicated treatments with either prednisolone or CsA, or PEG-IFN-α. The efficiency of HCV infection was assessed by qRT-PCR. Cells that were mock infected served as control. One patient serum of four is shown. Mean values of duplicate measurements including the error range are given.

Supplementary material

Figure S1: Time kinetic of prednisolone effect on SRB1 and occludin expression

Huh7.5 cells were treated with 100µg/ml prednisolone for four hours. Expression of SR-BI was determined by FACS analysis (A) or occludin by western blot (B) harvesting cells at the indicated time pointes during or post treatment with prednisolone.

Figure S2: The glucocorticoid receptor antagonist RU-486 has no influence on HCV infectivity alone

Huh-7.5 cells were inoculated with Luc-Jc1 virus particles prepared in the absence of any drugs. Indicated doses of RU-486 were added for 4 h, and infection efficiency was determined by luciferase assays 72 h post inoculation. Mean values of two independent experiments including the standard deviation are shown.

Figure S3: **Occludin and SR-BI mRNA levels are upregulated in primary human hepatocytes**

The mRNA levels of occludin, SR-BI and GAPDH were measured in primary human hepatocytes by qRT-PCR after 2, 6 and 10 days in the presence or absence of 100 µg/ml prednisolone.

Figure 1

Figure 2

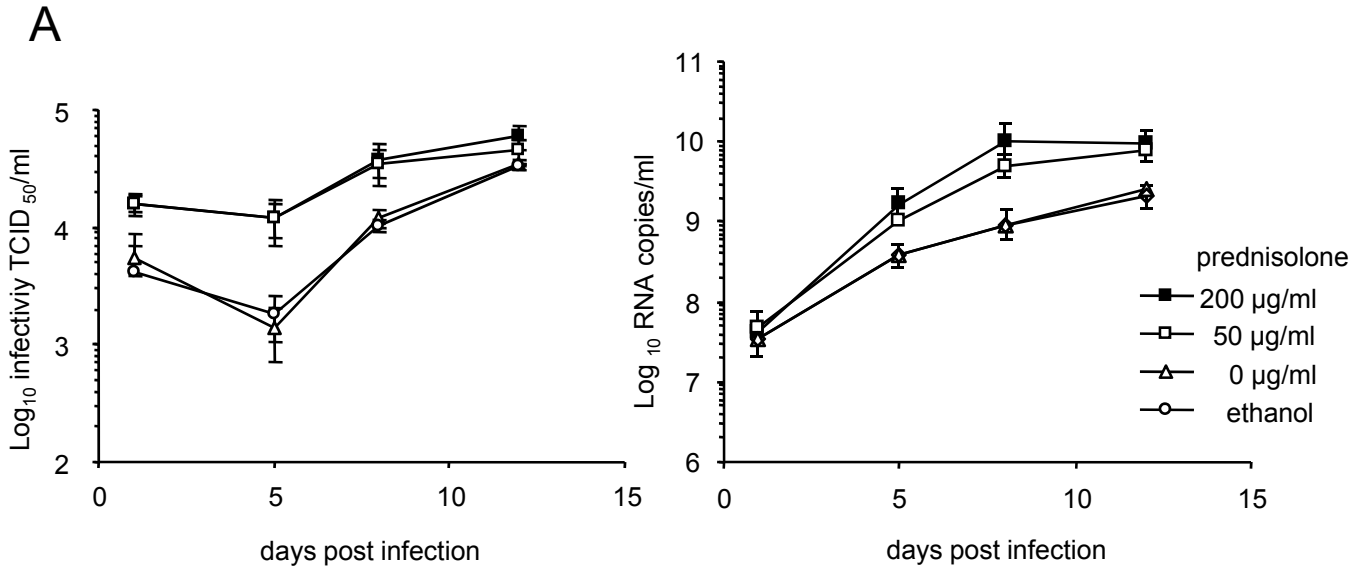**Figure 3**

A

B

Figure 4

A

B

Pretreatment with 10 µg/ml RU-486
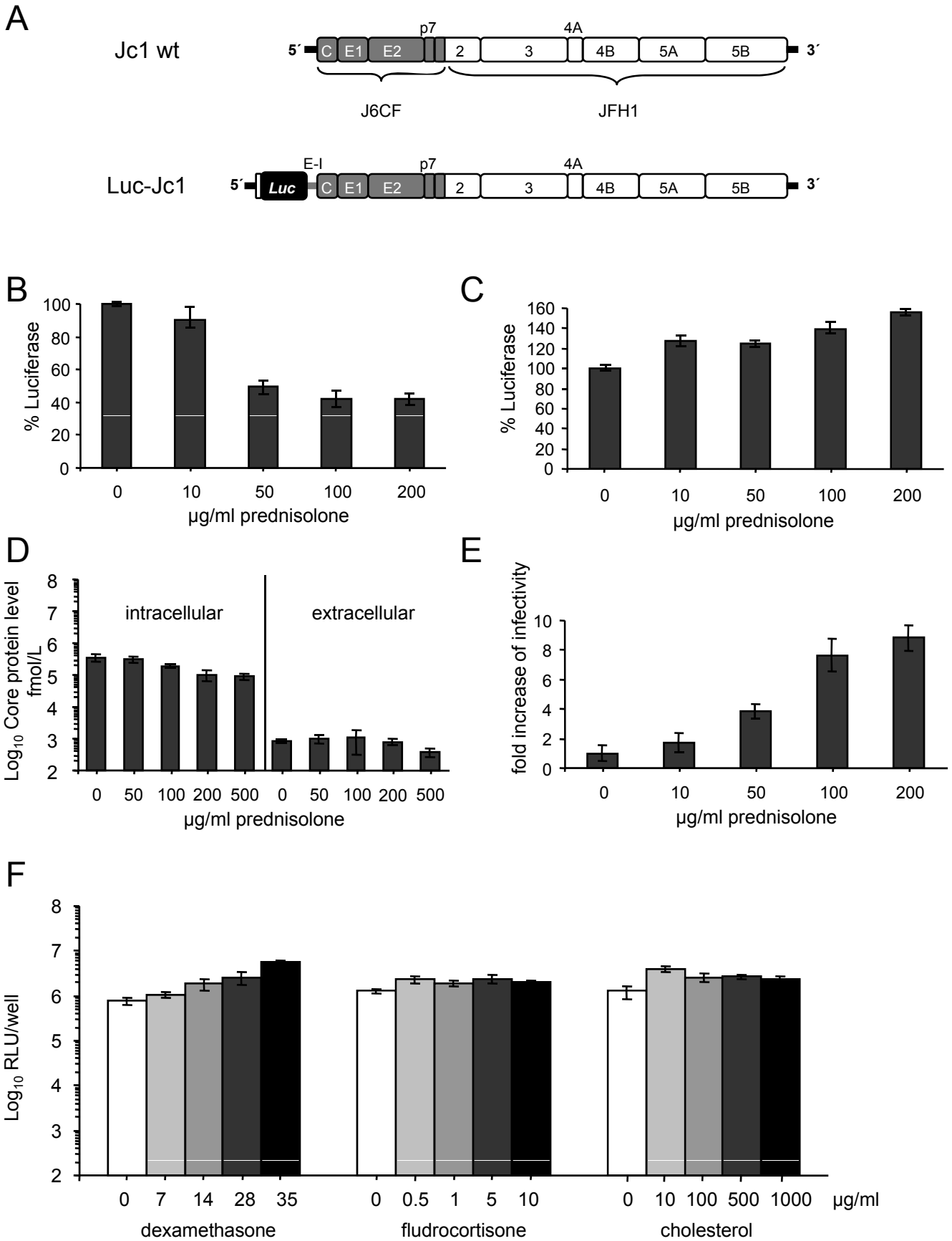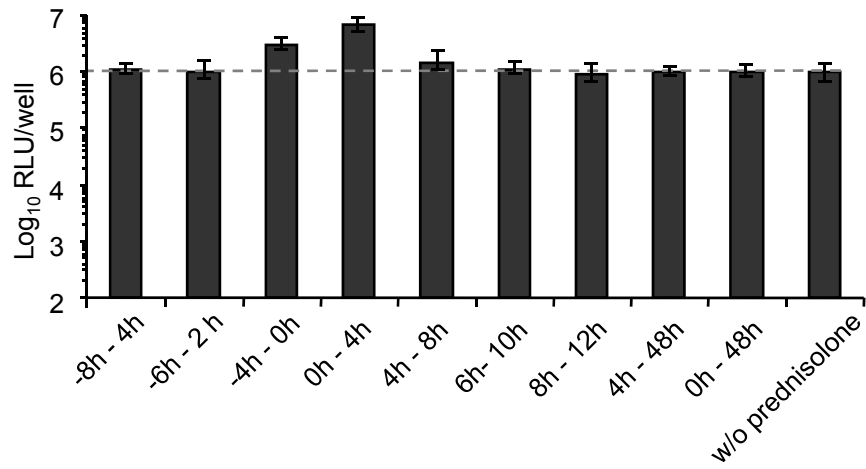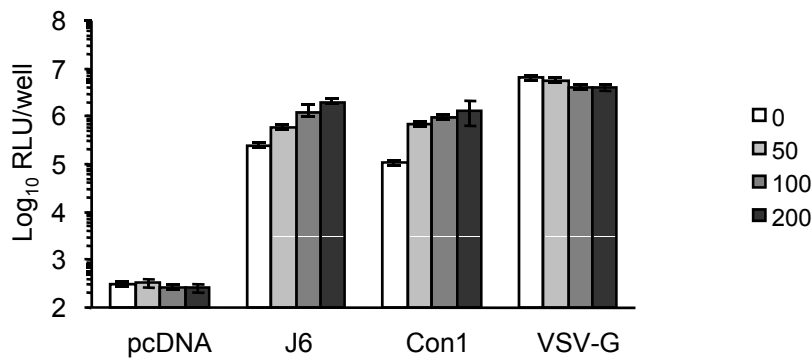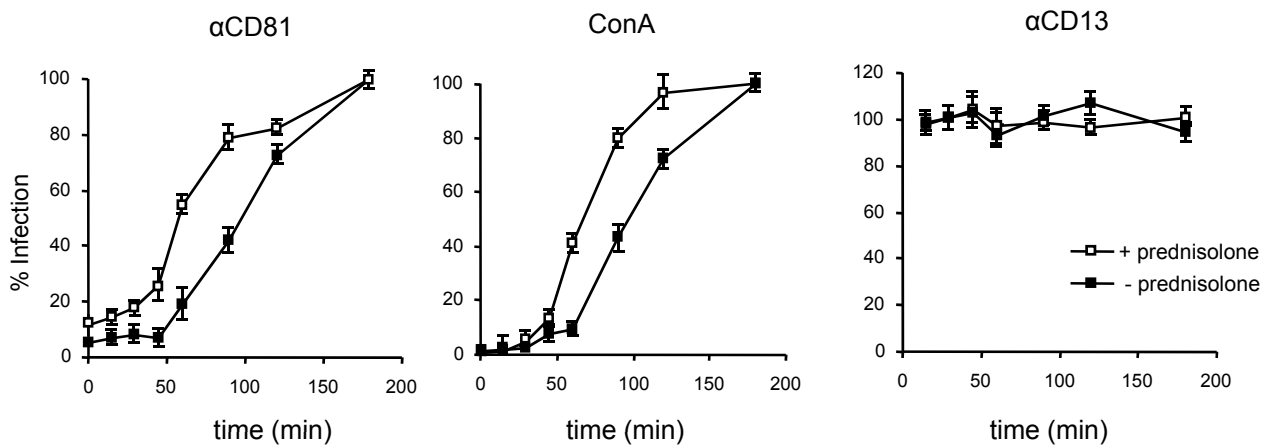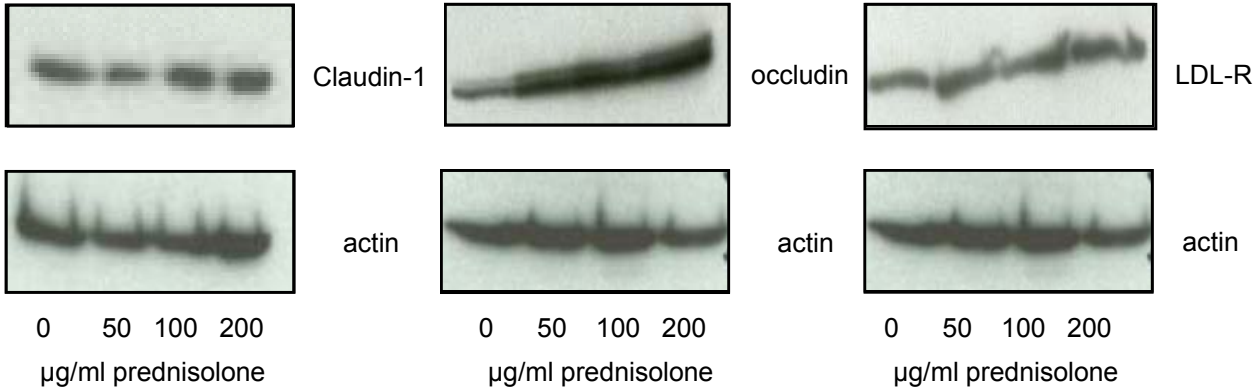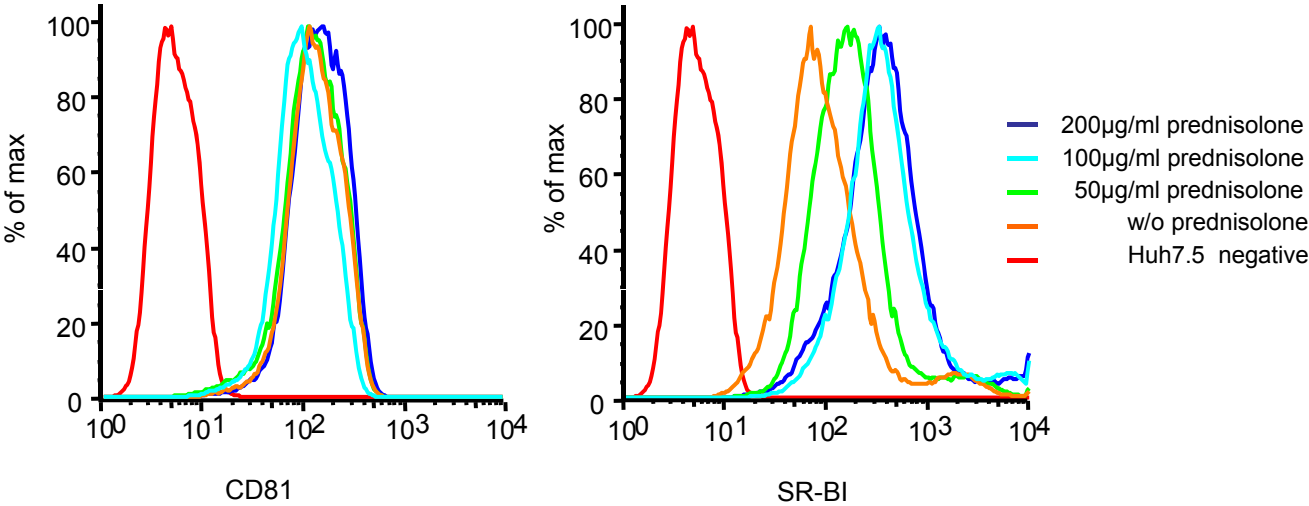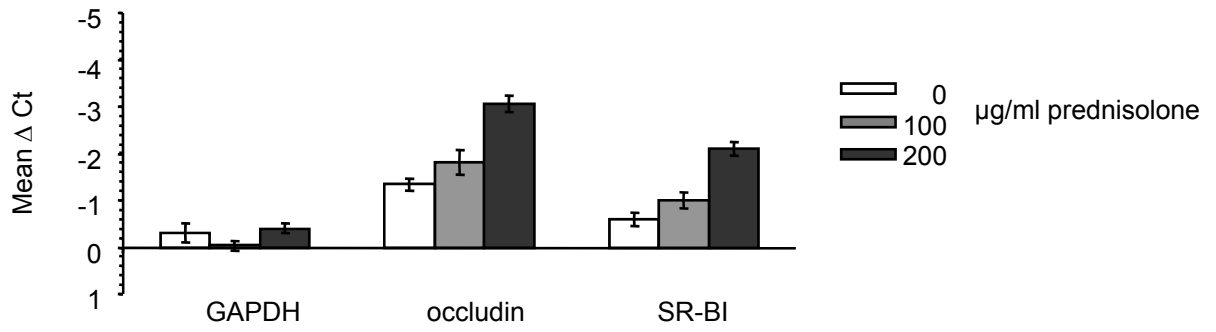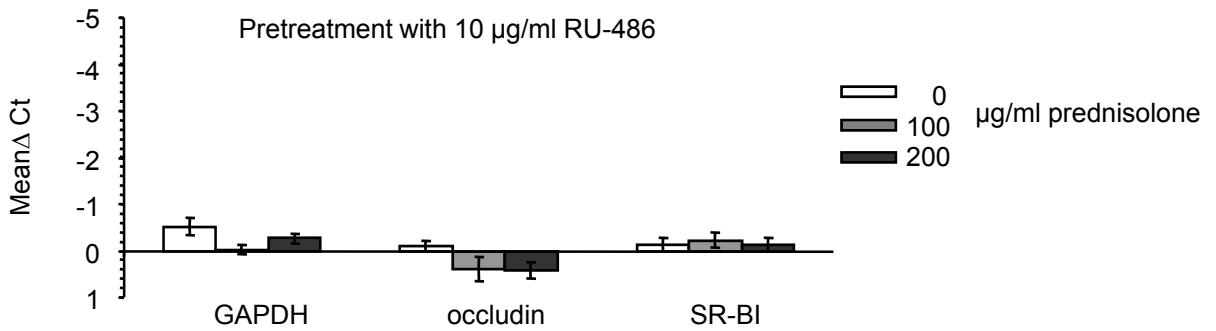
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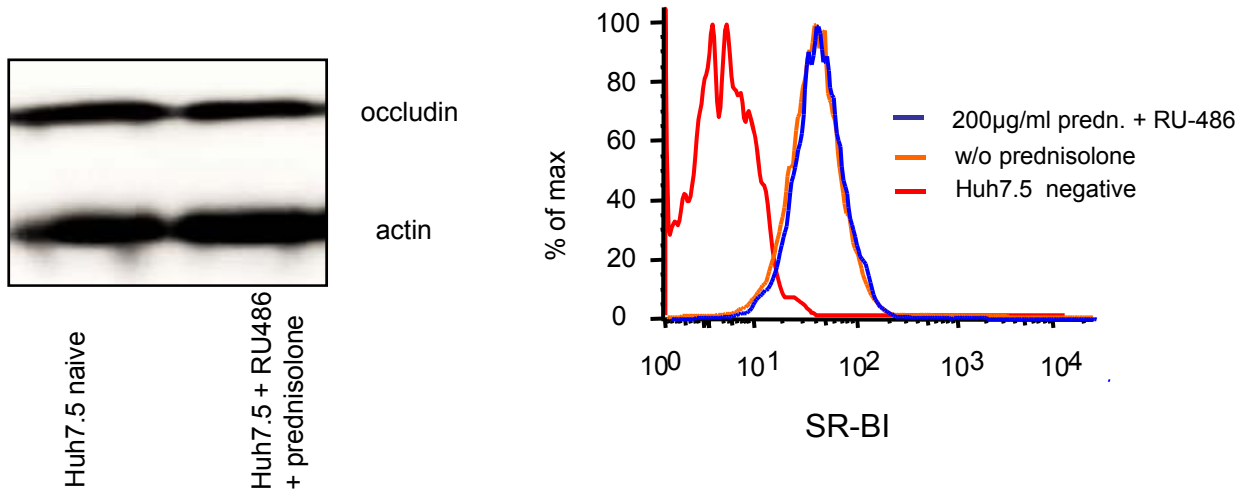
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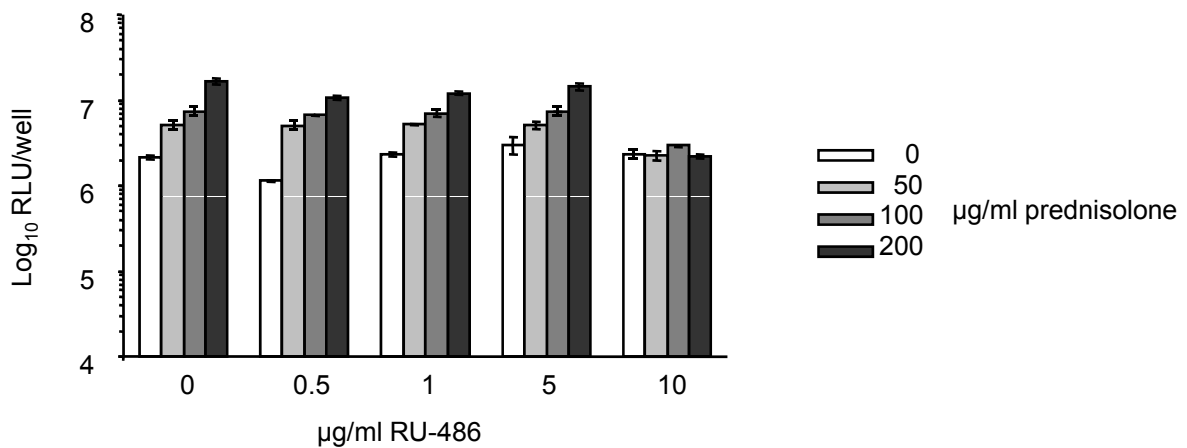
Figure 5

A
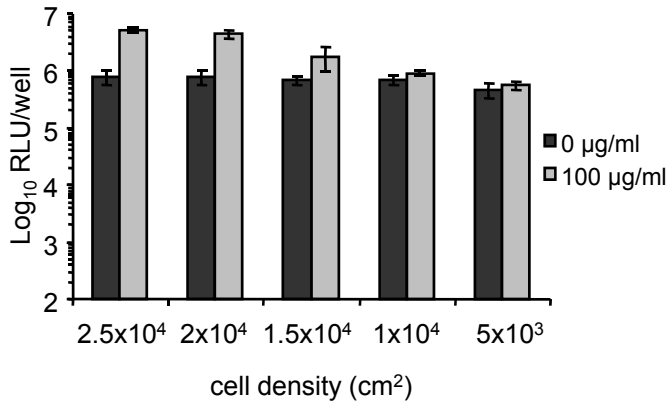
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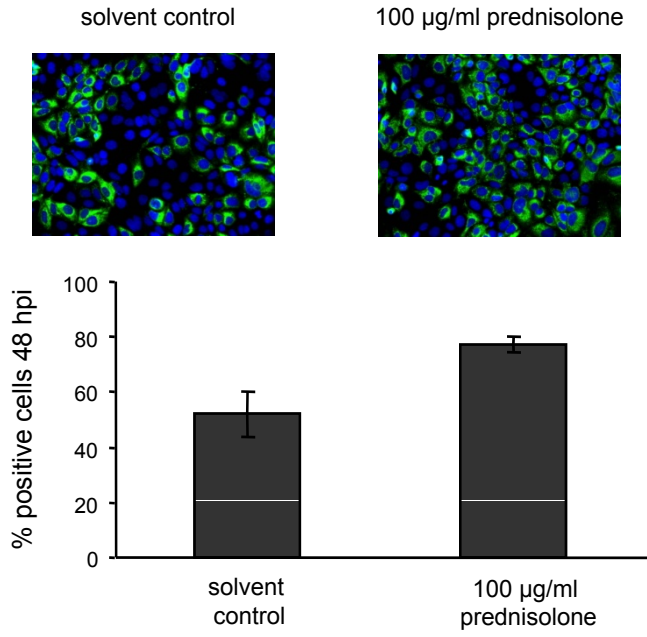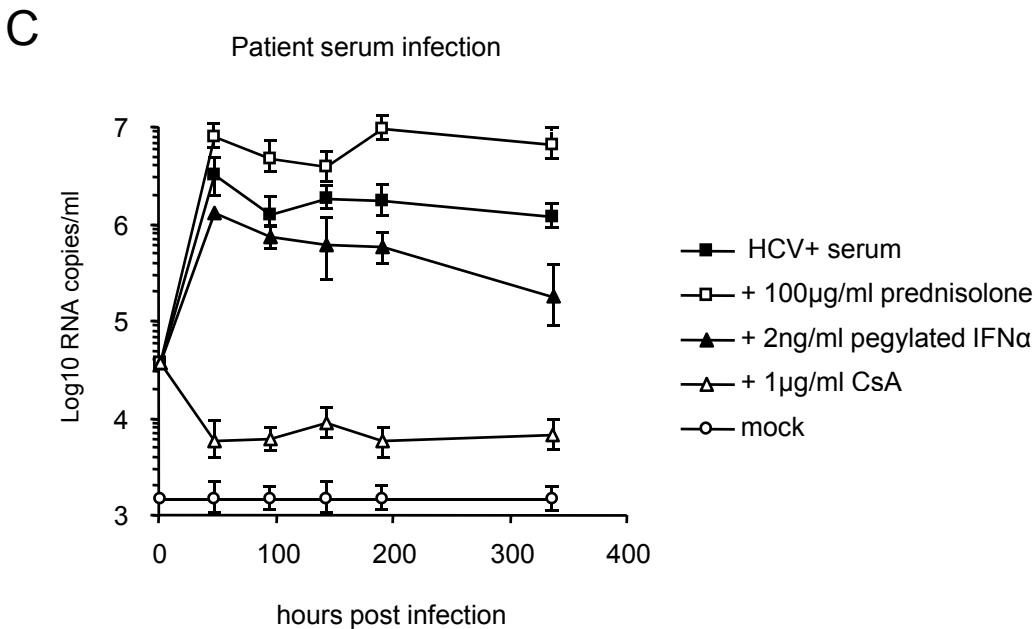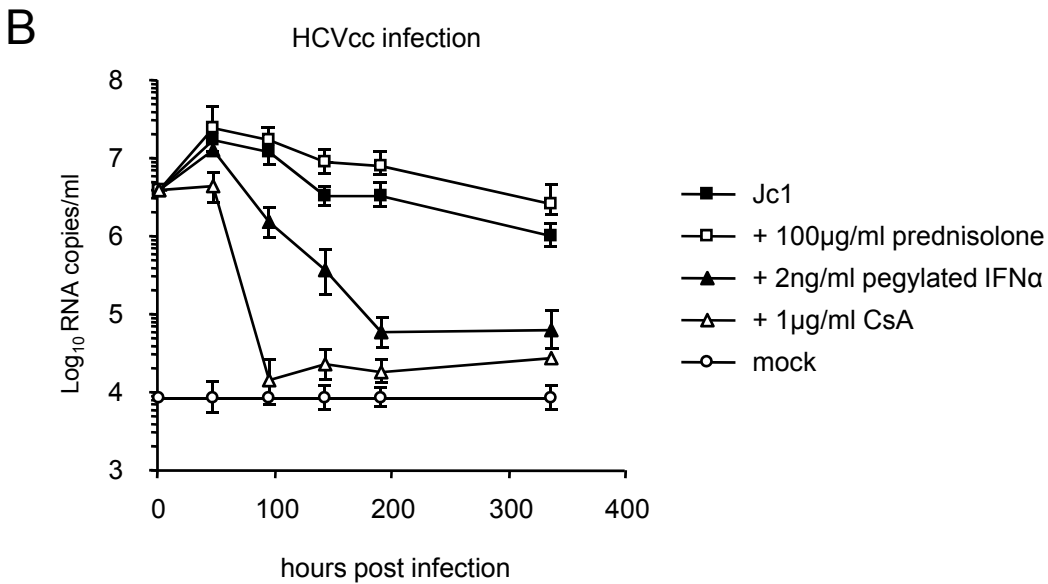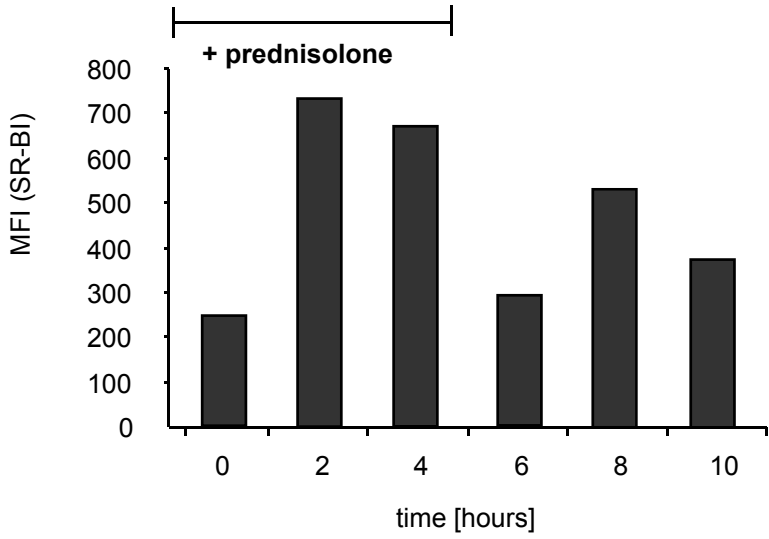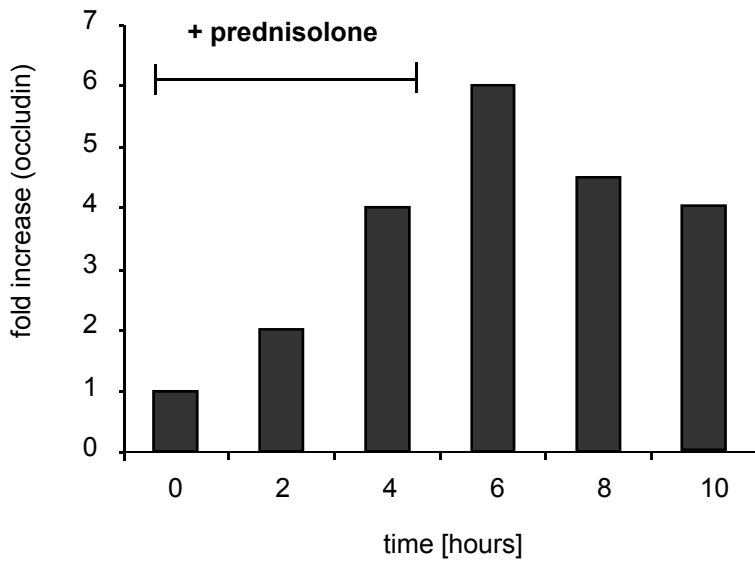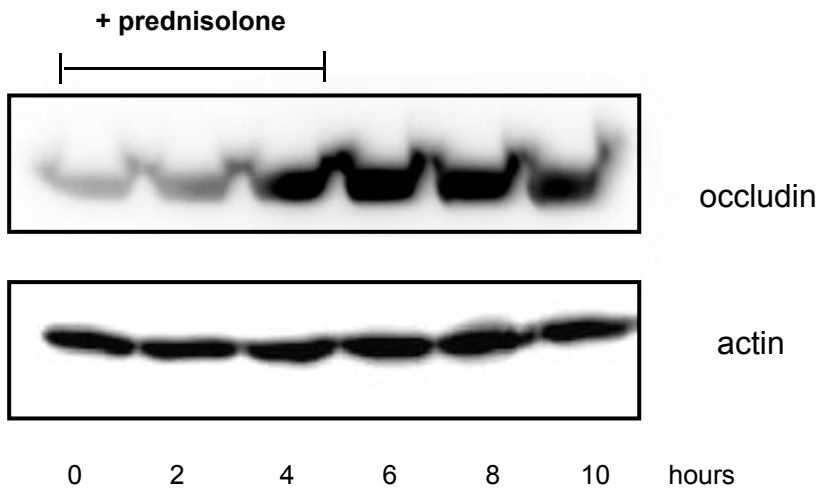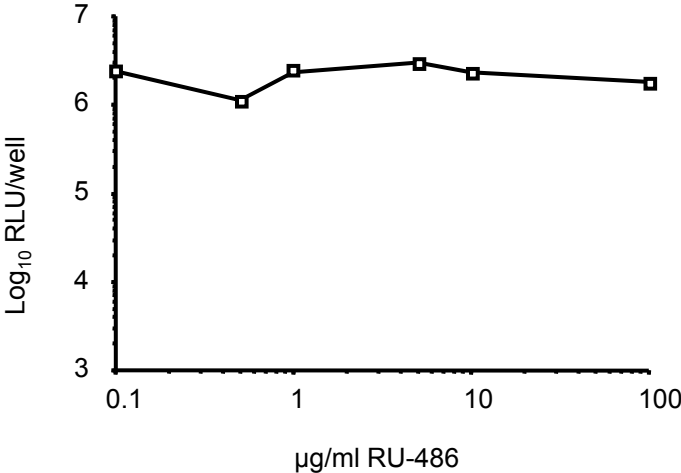
Figure 6

Suppl. Figure 1

A

B

Suppl. Figure 2

Suppl. Figure 3