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IL-26 inhibits hepatitis C virus replication in hepatocytes

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Conflict of Interest

The authors declare no competing interests.

Authors' contributions

EB and VL contributed equally to this work. PJ, PR and YD jointly supervised this work. EB, VL, LP, PP, SB, JD, CP, CM, MMP, AM, HF, PJ, PR and YD designed experiments. EB, VL, LP, PP, SB, BTH, JD, CP and CM performed experiments. EB, VL, LP, MMP, AM, HF, PJ, PR and YD interpreted data. MMP, PL, MP and HF provided reagents. EB, VL, PJ, PR and YD wrote the manuscript.

Data sharing

All data described in the manuscript can be shared on request.

ABSTRACT

Background & Aims: Interleukin-26 (IL-26) is a proinflammatory cytokine involved in the pathophysiology of chronic inflammatory disorders. It has properties atypical for a cytokine, such as direct antibacterial activity and DNA-binding capacity. In a previous study, we observed an accumulation of IL-26 in fibrotic and inflammatory lesions in the livers of patients with chronic hepatitis C virus (HCV) infection and showed that infiltrating $CD3^+$ lymphocytes were the principal source of IL-26. Surprisingly, immunohistochemical staining for IL-26 was also detected in the cytoplasm of hepatocytes from HCV-infected patients, even though these cells do not produce IL-26, even when infected with HCV. Based on this observation and possible interactions between IL-26 and nucleic acids, we investigated the possibility that IL-26 controlled HCV infection independently of the immune system.

Methods: We evaluated the ability of IL-26 to interfere with HCV replication in hepatocytes, and investigated the mechanisms by which IL-26 exerts its antiviral activity.

Results: We showed that IL-26 penetrated HCV-infected hepatocytes, where it interacted directly with HCV double-stranded RNA replication intermediates, thereby inhibiting viral replication. IL-26 interfered with viral RNA-dependent RNA polymerase activity, preventing the *de novo* synthesis of viral genomic single-stranded RNA.

Conclusions: These findings reveal a new role for IL-26 in direct protection against HCV infection, independently of the immune system, and increase our understanding of the antiviral defense mechanisms controlling HCV infection. Future studies should evaluate the possible use of IL-26 for treating other chronic disorders caused by RNA viruses, for which few treatments are currently available, or emerging RNA viruses.

Lay summary:

This study sheds new light on the body's arsenal for controlling HCV infection and identifies IL-26 as an antiviral molecule capable of blocking HCV replication. IL-26, which has unique biochemical and structural characteristics, penetrates infected hepatocytes and interacts directly with viral RNA, thereby blocking viral replication. IL-26 is, therefore, a new player in antiviral defenses, operating independently of the immune system. It is of considerable potential interest for treating HCV infection and other chronic disorders caused by RNA viruses for which few treatments are currently available, and for combating emerging RNA viruses.

Highlights:

- IL-26 is a new weapon in the antiviral arsenal, functioning independently of the immune system.
- IL-26 penetrates HCV-infected cells and interacts with viral double-stranded RNA replication intermediates.
- IL-26 interferes with viral RNA-dependent RNA polymerase activity, preventing the *de novo* synthesis of viral genomic single-stranded RNA.

INTRODUCTION

Interleukin-26 (IL-26) has been identified as a molecule overexpressed in human T cells transformed with saimiri herpesvirus,[1] and has been assigned to the IL-10 subfamily of cytokines.[1-3] However, IL-26 has unconventional physicochemical characteristics not found in other members of this family. It has an isoelectric point of 10.4, amphipathic properties and a predicted in-plane membrane (IPM)-anchoring motif reported to be involved in protein binding to cell membranes.[4,5] IL-26 resembles cationic cell-penetrating peptides designed for DNA delivery, accounting for its unusual functional properties. Indeed, we and others have reported that IL-26 binds extracellular DNA and shuttles DNA within myeloid cells[5] and plasmacytoid dendritic cells,[4] leading to the induction of type I interferons (IFN) and inflammatory cytokines. This activation involves DNA sensors and occurs in the absence of the heterodimeric receptor IL-10R2/IL-20R1, which was initially identified as a receptor for IL-26.[6]

IL-26 mRNA is expressed by activated Th1 and Th17 lymphocytes,[7,8] NK cells[8] and some non-immune cells (such as fibroblasts and smooth muscle cells) present in chronically inflamed tissues.[5,9] High serum IL-26 concentrations and IL-26 expression at sites of inflammation have been reported in patients with chronic inflammatory diseases, such as Crohn's disease,[7,8] rheumatoid arthritis[9] and vasculitis.[5,10] IL-26 has, thus, emerged as a link between cell death and persistent inflammation, and has been implicated in the pathophysiology of chronic inflammatory disorders.

We suspected that, in addition to this harmful role, IL-26 might have protective physiological functions, particularly in antiviral protection. This hypothesis is supported by previous studies. IL-26 modulates the infection of epithelial cells with vesicular stomatitis virus (VSV), human cytomegalovirus (HCMV), and herpes simplex virus type 1 (HSV-1).[11] We found that

treatment-naive patients with chronic hepatitis C virus (HCV) infection had high serum IL-26 concentrations, which were correlated with the degree of liver inflammation.[12] We also found that IL-26 increased the cytotoxic activity of NK cells against HCV-infected cells. We observed an accumulation of IL-26 protein in the cytoplasm of hepatocytes from patients with chronic HCV infection, in the absence of IL-26 mRNA detection.[12] As hepatocytes are the primary site of HCV replication, we suspected that the IL-26 accumulating in hepatocytes might have a direct antiviral effect. We report here that IL-26 inhibits HCV infection by interacting with the viral RNA and blocking its replication in hepatocytes. This result sheds new light on the antiviral defense mechanisms involved in controlling HCV infection and identifies IL-26 as a novel antiviral molecule.

MATERIALS AND METHODS

Cytokines

Recombinant N-terminally His_6-tagged human IL-26 ($IL\text{-}26_{His}$) was produced in *Escherichia coli* and purified, as previously described.[1,11] $IL\text{-}26_{His}$ did not contain detectable levels of endotoxin or contaminating proteins.[5] Recombinant human IFN-α was obtained from Immunotools (Friesoythe, Germany) and recombinant human TNFα, IFN-γ, TGFβ, IL-10, IL-19 and untagged IL-26 were obtained from R&D Systems (Abingdon, UK).

In vitro HCV infection

Huh7.5 cells (2×10^6 cells), which support efficient HCV replication,[13] were left untreated or were treated for 40 hours with 1-1000 ng/ml IL-26, 100 ng/ml IL-19 or 100 ng/ml IFN-α. Cells were then infected for 12 hours with 2×10^4 focus-forming units (FFU; equivalent to a multiplicity of infection (MOI) of 0.006) of the genotype 2a HCV JFH-1 strain,[14] optimized by 10 cycles of infection in naive Huh7.5 cells, as previously described.[15] The inoculum was removed, and cells were washed and cultured in fresh medium. Under these conditions, the virus infected all cells within six days and had not induced significant cytopathic effects by the end of replication. In some experiments, treatments were maintained until day 9 post-infection. In other experiments, treatments were imposed on the cells only after infection, and were maintained throughout the duration of infection. Cell pellets were collected at various time points post-infection (6 hours and days 1, 2, 3, 6, 9, and 12) for the quantification of intracellular HCV RNA (detailed in the supplementary materials and methods) and an evaluation of mRNA expression.

Analysis of mRNA expression

The expression of transcripts encoding molecules involved in virus entry (claudin-1 (CLDN1), scavenger receptor class F member 1 (SCARB1), CD81, occludin (OCLN), interferon-induced transmembrane proteins 1 and 3 (IFITM1 and IFITM3)), viral replication (guanylate-binding protein 1 (GBP1), interferon-regulated resistance GTP-binding protein (MxA), 2'-5'-oligoadenylate synthetase 1 (OAS1) and interferon-alpha-inducible protein 27 (IFI27)) or viral mRNA translation (interferon regulatory factor 7 (IRF7), DExD/H-Box helicase 58 (RIG-I))[16,17] was analyzed by RT-qPCR at various time points. The expression of CXCR4 mRNA was used as a positive control for JFH-1 replication.[18,19] The experimental procedures are described in the supplementary materials and methods.

Culture of human hepatocytes

Primary human hepatocytes cultured in collagen-coated plates in accordance with the manufacturer's recommendations (Life Technologies), were (i) stimulated for 24 hours with 100 ng/ml TNFα, 100 ng/ml IFN-γ, 450 μM etoposide (Sigma-Aldrich), or 100 ng/ml LPS (*E. coli* serotype O111:B4; Sigma-Aldrich) plus 1 μg/ml Pam3CSK4 plus 5 μg/ml CL097 (both from Invivogen, San Diego, CA), (ii) exposed to hypoxia or (iii) cultured for 3 days with 5 ng/ml TGFβ.

HCV pseudoparticle entry assays

HCV pseudoparticles (HCVpp) harboring the HCV envelope E1 and E2 glycoproteins derived from genotype 1a, 1b, 2a, 3a and 4 isolates or the VSV glycoprotein (VSV-G) were used to assess the capacity of IL-26 to prevent HCV entry. This assay is described in the supplementary materials and methods.

Confocal microscopy

The subcellular distributions of IL-26, HCV double-stranded RNA (dsRNA) and HCV NS5B were assessed by confocal microscopy, as described in the supplementary materials and methods.

Identification of potential RNA-binding sites on IL-26

A virtual 3D model of IL-26 was constructed with Modeller software, and validated with Procheck.[5] KYG software was used to identify potential RNA-binding residues.[20]

***In vitro* RNA replicase assay**

The capacity of IL-26 to modulate HCV RNA replication was assessed *in vitro*. In the absence of a functional RNA-dependent RNA polymerase (RdRp) for cell-free assays, we used the bacteriophage Phi6 RdRp as a surrogate model for HCV replicase. Indeed, these RdRps are structurally similar[21,22,23] and both use primer-independent *de novo* initiation mechanisms.[24,25] Our approach can therefore be considered realistic. This assay is described in the supplementary materials and methods.

***In vitro* interaction of IL-26 with RNA**

The interaction of IL-26 with HCV single-stranded RNA (ssRNA) and dsRNA was evaluated in a solid-phase binding assay described in the supplementary materials and methods.

Replicon assay

The Huh7.5-C5 cell clone was generated by the transfection of Huh7.5 cells with *in vitro* transcripts of the linearized pSGR-JFH-1 plasmid,[26] which contains the nucleotide sequence of the JFH-1 strain subgenomic replicon and a neomycin resistance gene, with selection on the basis of G418 resistance.[27] We used these cells to assess the capacity of IL-26 to inhibit

HCV replication in cell culture. Huh7.5-C5 cells (1×10^6 cells) were left untreated or were treated for 24, 48 or 72 hours with IL-26 (1, 10, 100 or 1000 ng/ml), IL-19 (100 ng/ml) or IFN-α (100 ng/ml). After treatments, cell pellets were collected for the quantification of intracellular HCV RNA, as described in the supplementary materials and methods. The results of three independent experiments performed in quadruplicate are shown, expressed as mean percent replication relative to mock conditions ± SEM.

RESULTS

IL-26 reduces HCV replication in cell culture

We first evaluated the ability of IL-26 to interfere with the *in vitro* replication of HCV. Huh7.5 cells were left untreated or were incubated with IFN-α (used as a control for protection) or various concentrations of IL-26 for 40 hours before infection with the HCV genotype 2a strain JFH-1. Cytokines were added to the culture medium daily until day 9 post-infection. IL-26 efficiently controlled HCV replication *in vitro*, in a dose-dependent manner, as shown by comparison with untreated infected cells. This control was particularly effective for IL-26 concentration ≥ 10 ng/ml, although IL-26 was unable to clear HCV completely (Fig. 1A).

In Huh7.5 cells treated once with IL-26 (particularly for concentrations of 100 or 1000 ng/ml) or IFN-α, 40 hours before infection, both cytokines were found to control HCV replication, resulting in viral loads significantly lower than those of untreated infected cells on day 3 post-infection (Fig. 1B). However, the protective effect of IL-26, unlike that of IFN-α, was transient, with viral load gradually increasing over time, suggesting that IL-26 and IFN-α have different mechanisms of action (Fig. 1B). Consistent with this observation, no protection was observed when IL-26 was added after HCV infection, regardless of the concentration used (Fig. S1). This result suggests that IL-26 cannot control an established infection, at least in the case of a highly replicative virus like the optimized JFH-1 strain. Finally, IL-19, another member of the IL-10 cytokine family, did not reduce HCV replication *in vitro*, whatever the protocol used, suggesting that the protective activity of IL-26 is not common to other IL-10 family members (Fig. 1A,B and Fig. S1). Thus, IL-26 can control HCV replication in Huh7.5 cells only when present throughout the replication period, highlighting the existence of a protective mechanism different from that mediated by type I IFNs.

We then investigated whether human hepatocytes produced IL-26 in response to stress (hypoxia, etoposide), or signals inducing inflammation (TLR agonists, inflammatory cytokines) or epithelial-mesenchymal transition (EMT). We found that etoposide, hypoxia, LPS + Pam3CSK4 + CL097, TGFβ1 (an EMT-inducer) and, as previously reported,[12] TNFα + IFN-γ did not induce IL-26 mRNA expression (Fig. S2). As controls, etoposide increased CD54 mRNA, TGFβ1 increased ANGPT1 mRNA, and LPS + Pam3CSK4 + CL097, TNFα + IFNγ, and hypoxia increased IL-8 mRNA expression (Fig. S2). We also confirmed that JFH-1 did not induce IL-26 mRNA expression in Huh7.5 cells. As a control,[18] the expression of CXCR4 mRNA increased in infected Huh7.5 cells (Fig. S2).

IL-26 does not prevent the entry of HCV into hepatocytes

We then investigated the mechanisms by which IL-26 exerts its antiviral activity. We first evaluated the capacity of IL-26 to interfere with the entry of HCV into Huh7 cells, using pseudotyped HIV-1-based retroviruses harboring HCV envelope glycoproteins derived from genotype 1a, 1b, 2a, 3 or 4 viruses. Whatever the duration of incubation, IL-26, IL-19 and IFN-α were unable to prevent the entry of HCVpp (Fig. 2A and 2B). Conversely, as reported for VSV-Gpp,[11] IL-26 slightly increased HCV entry into Huh7 cells for long incubation periods (40 hours). As expected,[28] a neutralizing mAb targeting the HCV membrane receptor CD81 markedly inhibited HCVpp entry (Fig. 2A and 2B). In support of these results, we also evaluated the capacity of IL-26 to inhibit the infection of hepatocytes with HCVpp and HCV in cell culture (HCVcc) previously exposed to IL-26. The results unambiguously showed that IL-26 was unable to prevent the entry of HCV into hepatocytes (Fig S3A and S3B). In conclusion, IL-26 does not affect the entry of HCV into target cells, suggesting that its protective effects against HCV infection involve the targeting of later stages of viral infection.

IL-26 does not induce the expression of antiviral genes

We then investigated whether the ability of IL-26 to control HCV infection was due to the induction of antiviral molecules. We analyzed the expression of genes encoding molecules involved in virus entry (CLDN1, SCARB1, CD81, OCLN, IFITM1 and IFITM3), virus replication (GBP1, IFI27, MxA, OAS1 and IRF7) and viral mRNA translation (IRF27 and RIG-I).[16,17] Huh7.5 cells have defective TLR3 and retinoic acid-inducible gene I (RIG-I) pathways and are therefore unable to sense viral nucleic acids.[29] Consequently, infection does not induce the expression of IFNs in Huh7.5 cells. We found that IL-26 did not induce expression of the ISGs IFITM1, IFITM3, MxA, OAS1, RIG-I, GBP1 and IFI27 in HCV-infected Huh7.5 cells. Moreover, the expression of mRNA encoding OCLN, CLDN1, SCARB1, CD81 and IRF7 was not modulated by IL-26 (Fig. 3). These findings are consistent with the dependence of the induction of ISGs by IL-26 complexed to nucleic acids on the induction of type I IFN by cells competent for nucleic acid sensing.[4,5] As expected,[29] IFN-α induced the expression of the ISGs IFITM1, IFITM3, MxA, OAS1, RIG-I, GBP1 and IFI27 mRNAs in HCV-infected Huh7.5 cells, demonstrating the functionality of the ISG-inducing pathways in these cells (Fig. 3). By contrast, the expression of SCARB1, CD81, OCLN1, CLDN and IRF7 mRNA was not modulated by IFN-α (Fig. 3). These results suggest that IL-26 controls HCV in Huh7.5 cells by a mechanism independent of ISG expression and of molecules involved in virus entry, translation or replication.

IL-26 binds to viral RNA and inhibits its replication

IL-26 is a cationic protein capable of binding to DNA.[4,5] We thus hypothesized that it might also interact with viral RNA, thereby interfering with viral replication. We first investigated the accumulation of IL-26 and viral dsRNA in Huh7.5 cells with and without IL-26 pretreatment. Confocal microscopy revealed that IL-26 accumulated in the cells in a time-

dependent manner, as demonstrated by the increase in fluorescence intensity corresponding to perimembranous labeling observed over time (Fig. 4 and Fig. S4). The punctate staining for IL-26 in the cytosol of infected cells colocalized with viral dsRNA staining ($R = 0.77 \pm 0.05$), suggesting a potential interaction between IL-26 and viral dsRNA at the endoplasmic reticulum. The occurrence of such an interaction was confirmed indirectly, by showing that IL-26 was partly colocalized with HCV NS5B, which operates at sites of viral replication ($R = 0.39 \pm 0.04$; Figure S5). Consistent with these findings, a structural model predicted an RNA-binding site within helix E of IL-26 (Fig. 5A). This prediction was confirmed by a solid-phase binding assay, showing that IL-26 bound to immobilized HCV RNA molecules, and that it preferentially bound dsRNA rather than ssRNA (Fig. 5B). IL-26 also bound VSV and SARS-CoV-2 RNA (Fig. 5B), suggesting that the binding of IL-26 to viral RNA is not dependent on specific nucleic acid sequences. We then explored the consequences of this interaction for *de novo* viral RNA synthesis *in vitro*, using the bacteriophage Phi6 RdRp as a surrogate model for the HCV replicase. We found that IL-26 and IL-26_{His} inhibited the synthesis of dsRNA, whereas BSA, used as a control protein, did not (Fig. 5C). IL-19, which also has amphipathic properties, inhibited the replicase activity of Phi6 similarly to IL-26 (IC50 = 13.0 and 12.1 ng/ml, respectively) (Fig. 5D). By contrast, IL-10 inhibited the replicase activity less strongly (IC50 = 87.2 ng/ml) (Fig. 5D). Finally, we evaluated the capacity of IL-26 to control the replication of the subgenomic replicon of JFH-1 in cell culture. Like IFN-α, IL-26 (used at a concentration of 100 or 1000 ng/ml) inhibited HCV replication in a dose-dependent manner, with an efficiency ranging from 60% for 48 hours of treatment to 95% for 72 hours of treatment (Fig. 5E). Consistent with our previous observations, IL-26 interfered with the last stage of HCV replication, by inhibiting the synthesis of new viral genomic ssRNA from the dsRNA replication intermediate.

DISCUSSION

We show here that IL-26 accumulates in HCV-infected cells and inhibits virus replication. These findings, together with the indirect antimicrobial properties (type I IFN induction and NK cell activation) of IL-26,[4,5,12] identify this cytokine as a novel antiviral molecule playing a major role in the control of HCV replication. Further studies are required to determine whether IL-26 can also inhibit the replication of other RNA viruses, and therefore has potential for use as a new therapeutic molecule.

An estimated 71 million people worldwide have HCV infection, which frequently leads to severe chronic liver disease. HCV infection is, therefore, one of the greatest challenges to human health of this decade. The recent introduction of direct-acting antiviral agents (DAAs) has considerably improved the treatment of chronic HCV infection,[30] but more than 390,000 people still die from HCV-related hepatic disease worldwide each year. Moreover, it has been estimated that the world reservoir of HCV-infected individuals increases by about two million subjects per year, and that this phenomenon is not limited to developing countries.[31] The eradication of this disease is hampered principally by the high cost of DAAs and by most HCV-infected subjects being unaware of their infection status (about 90% of the HCV-infected population worldwide), due to the asymptomatic nature of both acute and chronic infections.[32] Most of these individuals do not, therefore, receive treatment. Obtaining a full understanding of the antiviral defense mechanisms contributing to the control of HCV infection is therefore a major challenge.

The unique physicochemical characteristics of IL-26 result in unusual biological properties for a member of the IL-10 family, including DNA-binding capacity and antibacterial activity.[4,5] Since the discovery of IL-26 as a molecule overexpressed by human T cells transformed with saimiri herpesvirus,[1] questions have been raised about the potential antiviral role of IL-26. It has been reported that the preincubation of target cells or viruses with IL-26

modulates viral infectivity in various ways: it increases the infectivity of VSV, an RNA virus of the *Rhabdoviridae* family, decreases that of HCMV, and has no effect on the infectivity of HSV-1, these last two viruses being DNA viruses of the *Herpesviridae* family.[11] These results may reflect the capacity of IL-26 to interfere with the binding of the virus to membrane glycosaminoglycans. However, other than slightly increasing infectivity when used at high concentrations and in prolonged pretreatments, IL-26 had no effect on HCV entry into hepatocytes. These differences can be explained by the use of different viruses and *in vitro* models, and by the IL-26 concentrations used in the initial study, which were 10 to 50 times higher than those used here, and significantly different from those detected in infected patients.[12]

We previously reported that IL-26 accumulates in the cytoplasm of hepatocytes from patients with chronic HCV infection, and we identified infiltrating $CD3^+$ lymphocytes as the main IL-26-producing cells.[12] In support of this observation, we show here, by confocal microscopy, that IL-26 accumulates in the cytoplasm of IL-26-treated Huh7.5 cells, in a time-dependent manner. This accumulation can be accounted for by the biochemical and structural properties of this cationic molecule, which binds glycosaminoglycans on the surface of target cells, promoting its local accumulation.[6] The predicted IPM anchor may facilitate the interaction of IL-26 with cells.[5] Finally, IL-26 seems to penetrate cells thanks to its cationic and amphipathic properties, which are also displayed by cell-penetrating peptides,[33] such as LL-37.[34] The mechanism underlying the preferential accumulation of IL-26 in infected cells remains unknown, but may be related to changes in heparan sulfate proteoglycan levels in HCV-infected cells.[35,36] Finally, while it inhibited the replicase activity of Phi6 *in vitro*, IL-19 failed to protect Huh7.5 cells from HCV infection. This result was expected, as IL-19 has amphipatic features but, unlike IL-26, has no IPM anchor.[5]

The use of Huh7.5 cells made it possible to detect the inhibition of the viral replicase by Il-26 in the absence of type I IFN and ISG induction. Indeed, Huh7.5 cells do not express RNA sensors and do not display STAT3 phosphorylation in response to IL-26, despite expressing IL-10R2 and IL-20R1 mRNAs (Fig. S6). However, in physiological conditions, in addition to inhibiting viral replication, IL-26 probably exerts additional antiviral effects by inducing type I IFN and ISG expression in normal infected hepatocytes and by activating NK cells.

Based on its DNA-binding properties,[4,5] we hypothesized that IL-26 might also interact with viral RNA. Using the same 3D model of IL-26, we predicted the presence of a RNA-binding site within helix E. Confocal microscopy demonstrated the colocalization of HCV dsRNA, NS5B and IL-26 in HCV-infected Huh7.5 cells. The ability of IL-26 to interact with HCV RNA, and especially viral dsRNA, was also confirmed in a solid-phase binding assay. We also found that IL-26 was able to bind VSV and SARS-CoV2 RNA, suggesting that its viral RNA-binding capacity is not dependent on specific nucleotide sequences. IL-26 inhibited a viral replicase *in vitro*, preventing the production of new genomic ssRNA from dsRNA replication intermediates. In the absence of a cell-free assay mimicking HCV genome replication, the replicase Phi6 was used as a surrogate model for viral RdRps, with a *de novo* initiation mechanism.[23] Finally, using the JFH-1 strain subgenomic replicon, we confirmed the capacity of IL-26 to control HCV replication with an efficiency slightly lower than that of IFN-α.

In the HCVcc system, IL-26 was found to exert a protective role against infection when maintained at high concentration (100 ng/ml and especially 1000 ng/ml, Fig. 1), but had no effect, even at high concentration, if added after infection (Fig. S1). This result could be related to the high replication capabilities of the optimized JFH-1 strain used in these experiments. For this reason, we also evaluated the inhibitory properties of IL-26 on genotype 1a and 3a HCV strains with lower replication levels, both for its protective (pre- and post-

infection treatment) and curative (post-infection treatment) effects (Fig. S7). Results confirmed the protective role of IL-26 against genotypes other than genotype 2a and, importantly, confirmed that IL-26 exerts an inhibitory activity against established infections with strains exhibiting a low replication level. Taken together, these results suggest that IL-26 inhibits HCV replication by binding to its dsRNA replication intermediate, but also that this protective effect can be overwhelmed by a very high replication level.

IL-26 is cationic and amphipathic, like the cathelicidin antimicrobial peptide LL-37.[34] LL-37 enters cells[37] and exerts its antiviral properties via several mechanisms. LL-37 is virucidal against the vaccinia[38] and influenza A viruses[39], exerting this effect by altering viral membrane integrity. LL-37 also interferes with viral entry into host cells, as reported for varicella zoster virus[40] and dengue virus type 2.[41] LL-37 inhibits the *in vitro* replication of human immunodeficiency virus (HIV),[42] HCV[43] and respiratory syncytial virus,[44] independently of ISG induction. Wong *et al.* recently reported that LL-37 inhibits the HIV reverse transcriptase[45] via a mechanism similar to that described here. Nevertheless, IL-26 did not inhibit reverse transcriptase activity *in vitro*, and LL-37 did not inhibit the *in vitro* RNA replication mediated by Phi6 RdRp (data not shown). Thus, the ability of IL-26 to interfere with RNA replication is not common to all antimicrobial cationic peptides, and our findings suggest that specific elements, such as the nature of the virus and/or of the host cells, determine the specificity of the antiviral activity of antimicrobial molecules.

IFN-β was recently shown to have direct antimicrobial activities, in addition to its cytokine function.[46] Kaplan *et al.* suggested that IFN-β should be reclassified as a kinocidin, a recently identified family of molecules with both immune and direct antimicrobial properties. Based on the results reported by Meller *et al.* and our own results, we propose the reclassification of IL-26 as a kinocidin,[47] in light of its pro-inflammatory,[5,7-10,12] antibacterial[4] and, as reported here, antiviral properties.

In conclusion, we highlight a novel direct antiviral activity of IL-26 based on its ability to bind viral RNA and inhibit its replication. The antiviral activity of IL-26 was initially thought to be due to its capacity to induce type I IFN and to activate innate lymphoid cells. We show here that it may also provide direct protection against HCV infection.

Direct-acting antiviral molecules have been shown to cure hepatitis C in most patients. However, most clinical trials have focused on genotype 1a or 1b viruses, these two genotypes being the most common genotypes in Europe and the USA. Recent studies conducted in Western Europe demonstrated that migrant populations from developing countries of the South with a high prevalence of hepatitis C may be infected with unusual HCV subtypes that are resistant to DAA therapy.[48,49] In this context, the development of novel antiviral therapies, such as combinations of IL-26 with DAAs, could potentially improve the therapeutic management of these patients, especially in case of early infection and/or low level of viral replication. It will also be interesting to evaluate the potential protective role of IL-26 against other RNA viruses.

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FIGURE LEGENDS

Fig. 1. IL-26 reduces HCV replication in cell culture. Huh7.5 cells were left untreated or were treated for 40 hours with 1-1000 ng/ml IL-26, 100 ng/ml IL-19 or 100 ng/ml IFN-α. Cells were then infected for 12 hours with 2×10^4 FFU of an optimized HCV JFH-1 strain (MOI = 0.006). The inoculum was removed, and cells were washed and cultured in fresh medium. Treatments were either maintained until day 9 post-infection (A) or stopped at the time of infection (B). Cell pellets were collected at various time points post-infection (days 3, 6, 9 and 12) for the quantification of intracellular viral RNA by RT-qPCR. The results are expressed as absolute numbers of genome copies per ml (mean ± SEM, n=3). *** indicates statistically significant differences between infected Huh7.5 unexposed and those exposed to the different treatments after two-way ANOVA followed by Dunnett's test ($p < 0.001$).

Fig. 2. IL-26 does not prevent HCV pseudoparticle entry into Huh7 cells. HCV entry was evaluated with HCVpp generated with an HIV-1 luciferase reporter vector and harboring HCV envelope glycoproteins derived from genotype 1a, 1b, 2a, 3a and 4 isolates or the VSV glycoprotein (VSV-G). Huh7 cells were left untreated or were treated for 1 (A) or 40 hours (B) at 37°C with IL-26 (100 or 1000 ng/ml), IL-19 (100 ng/ml), IFN-α (100 ng/ml), anti-CD81 mAb (clone JS-81), or isotype control (both used at 5 µg/ml). Untreated and pretreated Huh7 cells were then infected by the different HCVpp for 6 hours at 37°C. The virus-containing medium was then removed and replaced with fresh medium. Infection levels were determined 72 hours post-infection, by measuring the luciferase activity of cell lysates. Results are expressed as mean percent entry relative to mock conditions (mean ± SEM, n=3). *, ** and *** indicate statistically significant differences between HCVpp-infected Huh7 untreated and those exposed to the different treatments after two-way ANOVA followed by Dunnett's test: $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Fig. 3. IL-26 does not increase the expression of ISGs and of genes involved in virus entry, replication and viral protein expression. Huh7.5 cells were left untreated or treated for 40 hours with 100 ng/ml IL-26 or IFN-α before infection with 2×10^4 FFU of an adapted HCV JFH-1 strain (MOI = 0.006); the treatment was maintained during the experiment. Levels of the mRNAs encoding IFITM1, IFITM3, SCARB1, OCLN, CLDN, CD81, MxA, OAS1, IRF7, RIG-I, GBP1 and IFI27 were evaluated by RT-qPCR at 6 hours and at days 1, 2, and 3 post-infection. Results are expressed as relative mRNA levels (mean ± SEM, *n*=3).

Fig. 4. IL-26 colocalizes with dsRNA in HCV-infected Huh7.5 cells. Huh7.5 cells were left untreated or were treated for 40 hours with IL-26 (1 μg/ml) before infection for 12 hours with 2×10^4 FFU of an optimized HCV JFH-1 strain (MOI = 0.06). The inoculum was removed, and cells were washed and cultured in fresh medium. After 72-hour incubation, during which IL-26 treatment (1 μg/ml) was maintained, the cells were fixed and processed for immunofluorescence analysis with DAPI, anti-dsRNA mAb or polyclonal anti-IL-26 Ab. The subcellular distribution of the protein was assessed by confocal microscopy. The colocalization of IL26 and HCV dsRNA was quantified with Imariscoloc (R = 0.77 ± 0.05). Scale bar, 20 μm.

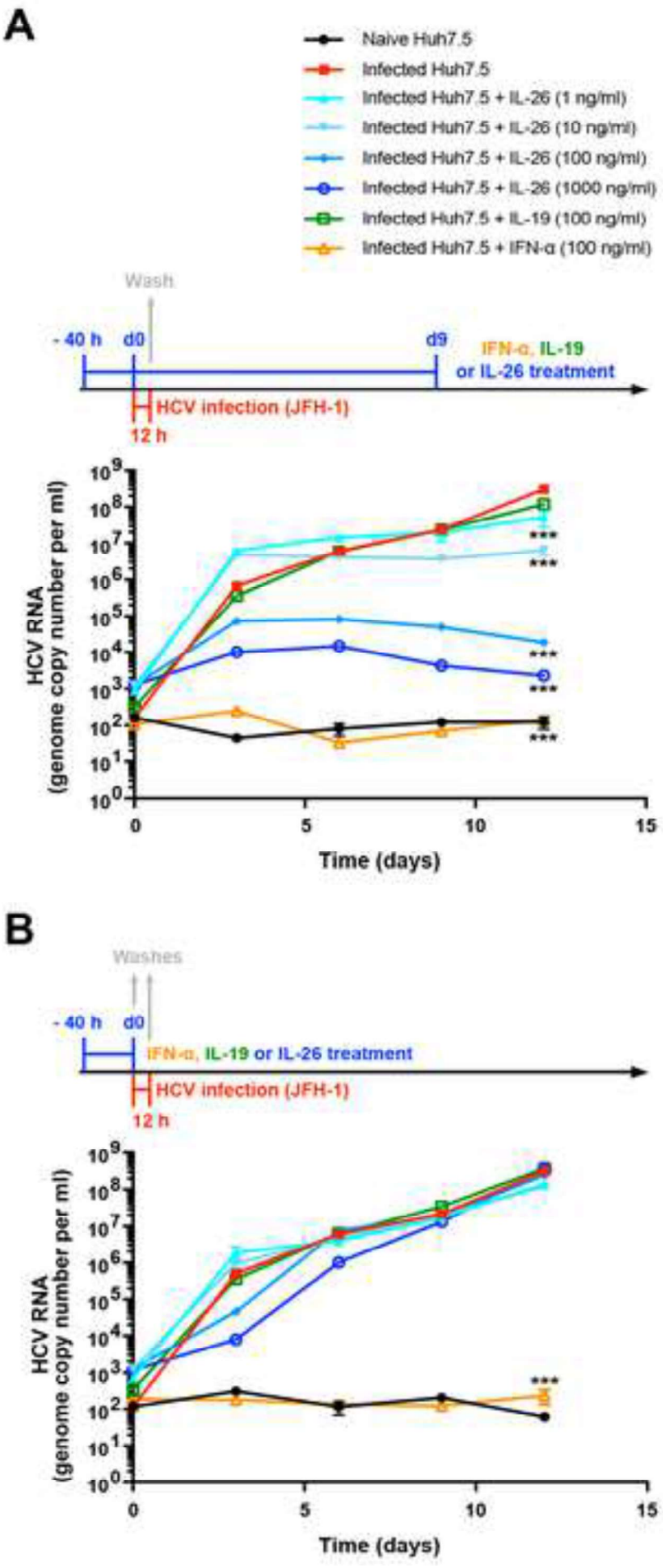
Fig. 5. IL-26 interacts with HCV RNA and inhibits its replication. (A) A three-dimensional structural model of IL-26 was generated *in silico*, using the crystal structure of IL-10 as a template. An RNA-binding site (black circle) was predicted by KYG software. The bar below represents the RNA-IL-26 binding probability, colored from low (red) to high probability (blue). (B) For RNA binding assay, HCV- (JFH-1), VSV- and SARS-CoV-2-specific dsRNA and ssRNA molecules were immobilized before incubation with 20 ng/ml IL-

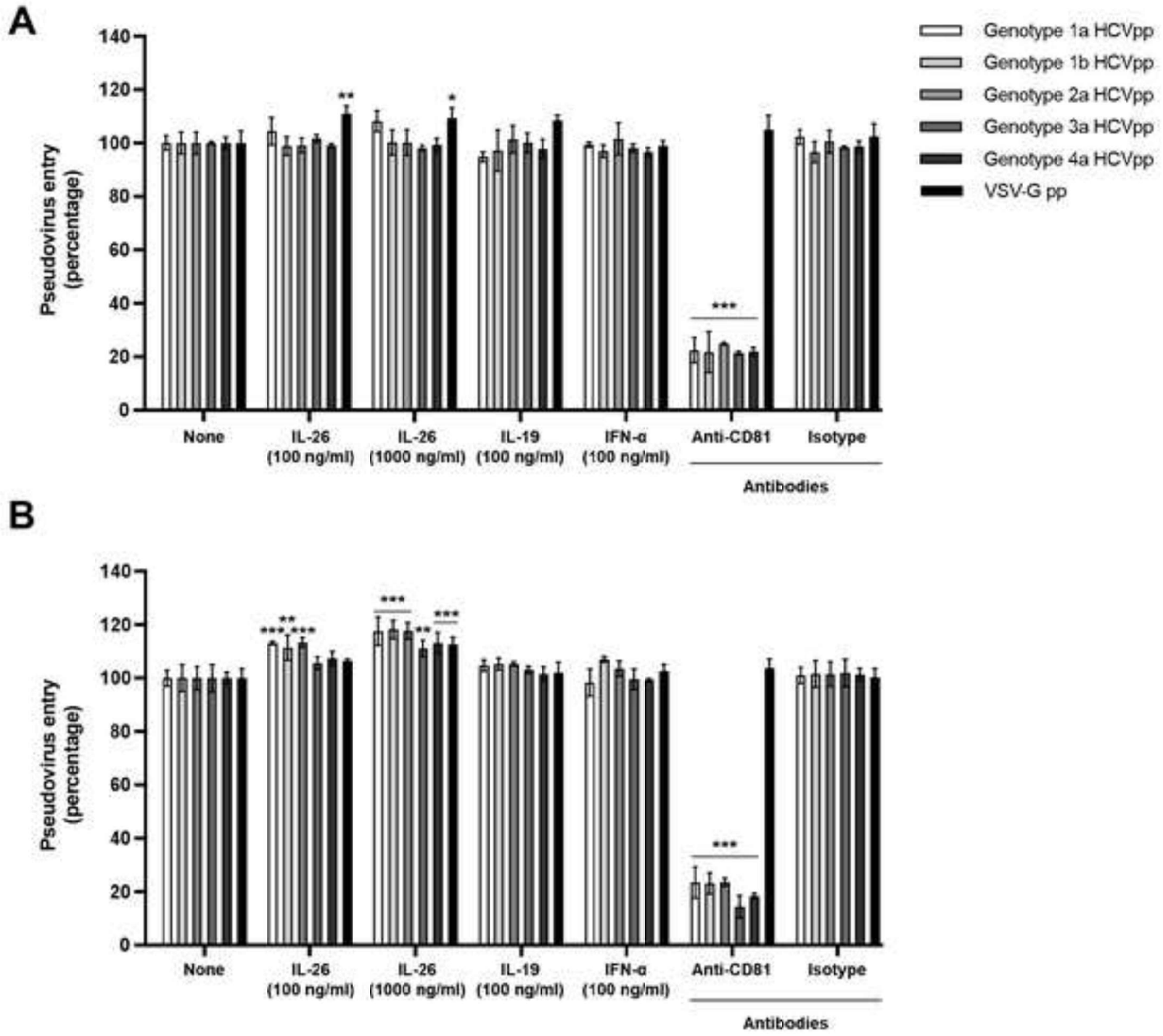
26. Bound IL-26 was detected by incubation with a biotin-labeled anti-IL-26 mAb followed by HRP-conjugated streptavidin. Results are expressed as optical density values (mean ± SEM, n=7; ***: p<0.001 (Mann-Whitney tests)) (C) HCV-specific ssRNA and dsRNA molecules (100 ng) were replicated *in vitro* with the Phi6 RdRp, in the presence or absence of 100 µg/ml (5 µg/well) tagged ($IL\text{-}26_{His}$) or untagged IL-26 or an equal amount of BSA. (D) HCV-specific ssRNA and dsRNA molecules (100 ng) were replicated *in vitro* with the Phi6 RdRp, in the presence or absence of 0.1-100 µg/ml IL-10, IL-19 and IL-26. (C&D) The replication was monitored by evaluating the incorporation of biotinylated CTP into the replication product; biotin-14-CTP was detected by incubation with HRP-labeled streptavidin. Results are expressed as optical density values (mean ± SEM, n=7; **: p<0.01 (Mann-Whitney tests)). (E) Huh7.5-C5 cells containing the JFH-1 strain subgenomic replicon were left untreated or were treated for 24, 48 or 72 hours with IL-26 (1, 10, 100 or 1000 ng/ml), IL-19 (100 ng/ml) or IFN-α (100 ng/ml). After treatments, cell pellets were collected for the quantification of intracellular HCV RNA by RT-qPCR. Results are expressed as mean percent replication relative to mock conditions (mean ± SEM, n=3). ** and *** indicate statistically significant differences between untreated Huh7.5-C5 cells and those exposed to the different treatments after two-way ANOVA followed by Dunnett's test: $p < 0.01$ and $p < 0.001$, respectively.

ABBREVIATIONS

ACTB	β-actin
CLDN1	Claudin-1
CTCF	Corrected total cell fluorescence
CXCR4	C-X-C chemokine receptor type 4
DAAs	Direct-acting antiviral agents
dsRNA	double-stranded RNA
EMT	Epithelial-mesenchymal transition
FFU	Focus-forming units
GBP1	Guanylate-binding protein 1
HCMV	Human cytomegalovirus
HCVcc	HCV in cell culture
HCVpp	HCV pseudoparticles
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSPCB	Heat shock protein 90 kDa beta
HSV-1	Herpes simplex virus type 1
IFI27	Interferon-alpha Inducible Protein 27
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IPM	In-plane membrane
IRF7	Interferon regulatory factor 7
ISG	Interferon-stimulated gene
LPS	Lipopolysaccharide
mAb	monoclonal antibody

MOI	Multiplicity of infection
MxA	Myxovirus resistance gene A
OAS1	2'-5'-oligoadenylate synthetase 1
OCLN	Occludin
OD	Optical density
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene I
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCARB1	Scavenger receptor class F member 1
ssRNA	single-stranded RNA
TBP	TATA-box binding protein
TGFβ	Transforming growth factor β
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	Tumor necrosis factor α
TRFC	Transferrin receptor 1
UTR	Untranslated region
VSV	Vesicular stomatitis virus
VSV-G	VSV glycoprotein
VSV-M	VSV protein M





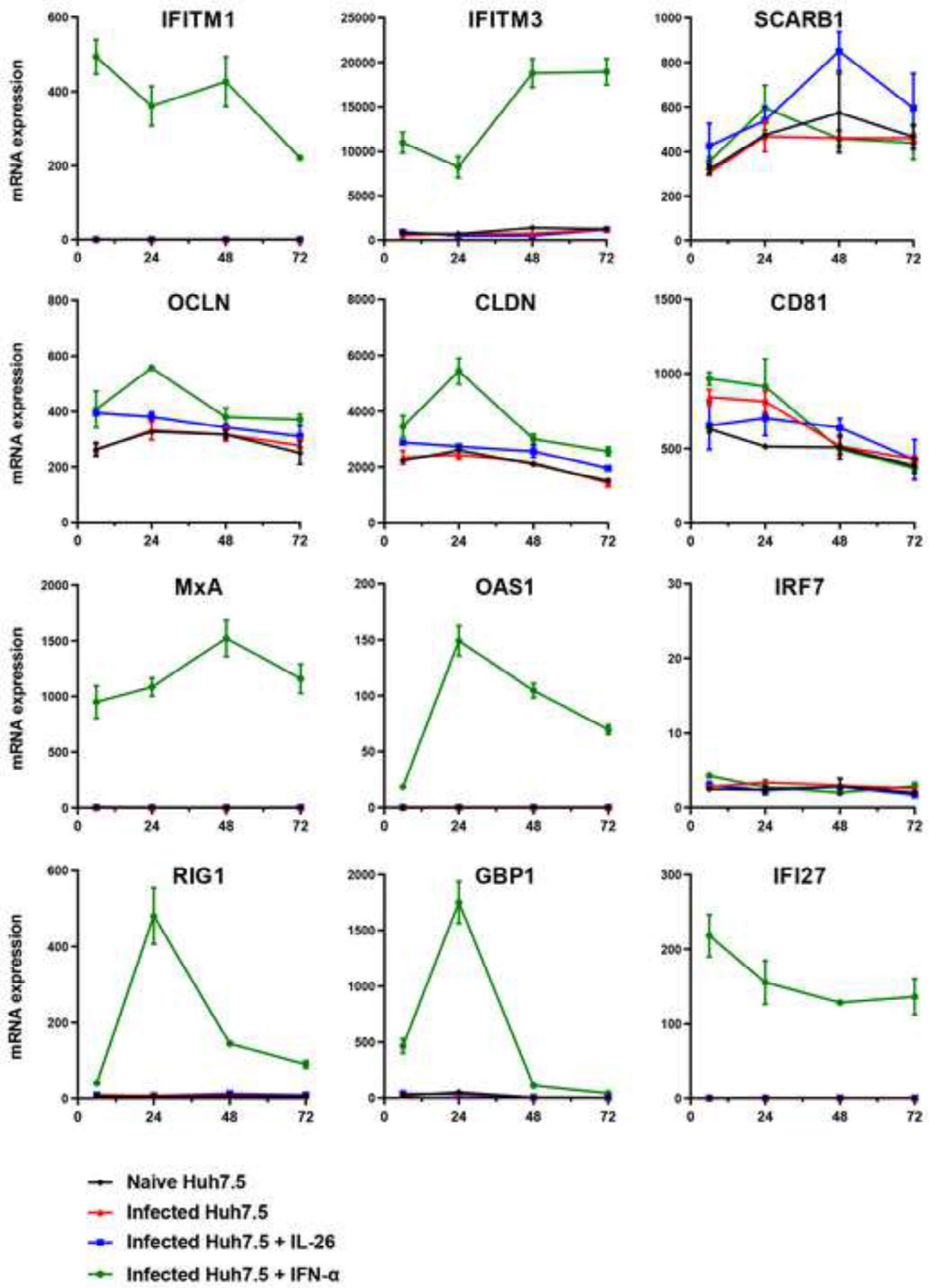


Figure 4