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RESEARCH ARTICLE

Modulation of IL-2 expression after uptake of hepatitis C virus non-enveloped capsid-like particles: the role of p38 kinase

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Abstract Hepatitis C virus (HCV) has been shown to actively replicate in cells of the immune system, altering both their function and cytokine expression. Naked nucleocapsids have been reported in the serum of infected patients. We investigated interference of recombinant non-enveloped capsid-like particles with signaling pathways in T cells. HCV non-enveloped particles (HCVne) internalization was verified in Jurkat and Hut 78 T cells, as well as primary human peripheral blood and intrahepatic mononuclear cells. HCVne uptake leads to activation of the MAPKs-p38 signaling pathway. Using specific phosphoantibodies, signaling pathways inhibitors, and chemical agents, it was demonstrated that p38 activation in T cells correlated with IL-2 transcriptional activation and was accompanied by a parallel increase of IL-2 cytokine secretion. *c-fos* and *egr-1*, two transcription factors, essential for IL-2 promoter activity, were also found to be elevated. We propose that HCVne uptake by T lymphocytes results in increased MAPKs-p38 activity and IL-2 expression, thus altering the host immune response.

Keywords Hepatitis C virus · Non-enveloped particles · IL-2 · p38 · T cells

Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped virus with a positive-sense RNA genome of 9.6 kb. The viral genome encodes for a single polyprotein that is subsequently processed by viral and cellular proteases to generate ten structural and non-structural polypeptides [1]. HCV was identified in 1989 and is now considered to be endemic worldwide, affecting at least 170 million people globally, or 3% of the world's population [2].

Although HCV is an enveloped flavivirus, only a minor population of serum-isolated particles has properties matching those of the canonical flavivirus-like particles. Instead, various unconventional forms of HCV were detected and characterized in HCV-positive sera [3]. It is of note that the lipid-associated core particles were associated with clinical remission of liver damage, while naked nucleocapsids were observed mainly in sera with active disease [4]. Furthermore, HCV subgenomes with in-frame deletions of both envelope proteins (E1 and E2) were

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identified with relatively high abundance in the liver as well as in the serum of HCV-infected individuals [5–8].

Among HCV-infected individuals, more than 70% develop chronic infection due to viral persistence, while 20% spontaneously clear the virus. There is compelling evidence that the outcome of primary HCV infection is dependent on the vigor and the type of the antiviral cell-mediated immunity [9]. Strong and sustained Th1 cytokine responses—which include the secretion of IL-2 and IFN-gamma (IFN-γ), have been reported to protect against chronic infection and promote viral clearance, while Th2 responses, along with relatively weaker and narrower cytotoxic T-lymphocyte response, may favor HCV persistence [10, 11]. Currently, the mechanisms underlying the failure to sustain peripheral T cell responses in chronic HCV infection can be classified into four main groups: escape, exhaustion, helplessness, and regulation, according to the different T cell subsets that develop after HCV infection [12]. These mechanisms include CD8+ and CD4+ T cell deletion, anergy, cytotoxic T lymphocyte exhaustion, and suppression via regulatory CD4+ CD25+ T cells and IL-10-secreting regulatory CD8+ T cells [13].

The mechanisms triggering the apparent downregulation of CD8+ and CD4+ T cell responses associated with persistent HCV infection are crucial in understanding HCV persistence. According to the model of T cell "partial" exhaustion, viral persistence is associated with decline and loss of CD8+ and CD4+ T cells, which results in a progressive loss of IL-2 secretion, T cell proliferation, IFN-γ secretion, and cytotoxicity occurring prior to full deletion [14]. In contrast, CD4+ T cells are observed in high levels in acute infection and are maintained in those who clear the virus. However, they are typically lost in cases where the virus is not cleared [12].

The regulation and production of IFN-γ as an immune response after HCV infection has been widely studied. Non-cytolytic effector mechanisms of CD4+ and CD8+ T cells that act to clear HCV-infected hepatocytes include the production of several cytokines, including IFN-γ, which has been demonstrated to inhibit HCV replication in some but not all model systems [15, 16].

Despite the fact that HCV is a hepatotropic virus, it has been shown to infect and actively replicate in cells of the immune system, altering their function and cytokine expression [17, 18]. In this way, HCV seems to interfere with both innate and adaptive immunity. Previous studies have reported replication of HCV in B cells, T cells, and peripheral blood mononuclear cells, besides the hepatocytes of HCV-infected individuals [17, 19–23]. Existence of HCV strains with preferential lymphocyte tropism suggests a potential role of T cells as an HCV reservoir [18]. Limited evidence suggesting that the HCV variants in immune cells of the patients with chronic hepatitis C are distinct from those in the plasma or the liver could imply that different cell types may promote propagation of tissue-specific virus variants. Furthermore, viral replication in T cells may directly affect T cell functions [17, 24].

We have previously reported the generation of recombinant non-enveloped HCV core particles (previously designated HCVne) [25] in the absence of other HCV proteins. In addition, we have demonstrated that these naked capsids can be uptaken by immune cells, such as T- and B-lymphocytes [26, 27].

The modulation of T cell responses is the hallmark of many persistent infections and is often associated with the expression of specific viral products. T cell activation through the T cell receptor (TCR)/CD3 complex and the CD28 costimulatory molecule results in enhanced production of the autocrine growth factor interleukin-2 (IL-2) [28, 29]. IL-2 exerts its effects on many cell types, and predominantly on effector T lymphocytes. Accordingly, a major function of IL-2 is to promote proliferation and expansion of both antigen-specific clones of CD4+ and CD8+ T cells as well as to induce production of other cytokines. In CD4+ T cells, this cytokine also stimulates differentiation to the Th2 subset [30], induces apoptosis of activated T lymphocytes, and plays a non-redundant role in the development of CD4+ CD25+ Tregs [31]. In CD8+ cells, IL-2 augments cytotoxic activity and activates proliferation of memory CD8+ cells [32]. Furthermore, IL-2 contributes to the production of IFN-γ by human lymphocytes, suggesting that a cascade of lymphocyte-cell interactions participates in human immune responses [33].

TCR triggering causes the activation of a complex array of proximal signals, resulting in calcium oscillations and protein kinase C/p21ras-mediated activation of two mitogen-activated proteins kinases (MAPK), extracellular regulated kinase (ERK), and p38. These molecular events are involved in the activation of transcription factors that bind at distinct sites on the IL-2 promoter such as nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), nuclear factor-κB (NFκB), and CRE-binding protein (CREB) [34].

Recently, p38 was reported to regulate IL-2 production in a stimulation-dependent manner [34]. It has also been reported that p38 positively regulates IL-2 gene expression in Jurkat T cells [35], SB203580, a specific p38 inhibitor, was shown to inhibit IL-2 production by murine T cells [36]. In contrast, studies with primary human T cells indicated that p38 inhibition has no or only a minimal positive effect on IL-2 production [37, 38], suggesting that the p38 MAPK has a polyvalent role in IL-2 expression. Hence, an interesting regulation model was proposed where p38 acts as a gatekeeper that controls the levels of ERK activation that sets the threshold for IL-2 expression [39]. Recently, it was also reported that p38 MAPK

negatively regulates IL-2 expression and promotes IL-2R signaling, which leads to T lymphocyte proliferation [40].

Previous studies have demonstrated that HCV core protein expression can regulate IL-2 expression in T cells [41, 42]. In the light of this and all the observations described above, our experiments were designed in order to investigate whether HCVne binding/uptake by T cells could activate MAPK-p38, thus leading to IL-2 regulation. Here we report that p38 is activated and subsequently promotes IL-2 expression after challenging of T cells with HCVne particles. ERKs are also activated and follow similar phosphorylation kinetic pattern with p38. We subsequently studied transcription factors, which are known to bind on IL-2 promoter and regulate its expression such as CREB, *c*-fos and egr-1 [43, 44]. In addition, the endogenous levels of IFN-γ were examined and found elevated in T cells challenged with HCVne, indicating that when HCVne enters T cells, it promotes activation and modulates cell-mediated immunity.

Materials and methods

Antibodies and reagents

The following antibodies were used: mouse monoclonal anti-actin (Chemicon), phospho-p44/42 rabbit monoclonal, rabbit anti-phospho p38, p38 mouse monoclonal, c-fos rabbit monoclonal, phospho-CREB mouse monoclonal (all from Cell Signaling), ERK1 (K-23) rabbit polyclonal which detects both ERK1 and ERK2, CREB-1 rabbit polyclonal, GFP rabbit polyclonal (all from Santa Cruz), HCV core mouse monoclonal antibody (Alexis Biochemicals) and HCV core rabbit polyclonal antibody [27]. As secondary antibodies for immunostaining, we used rabbit and mouse Alexa Fluor 488-conjugated antibodies (Molecular Probes). The p38 inhibitor SB203580, Phorbol 12-myristate 13-acetate (PMA), ionomycin, anisomycin and forskolin were all purchased from Sigma. MEK inhibitor UO126 was from Cell Signaling. 7-Actinomycin-D (7-AAD) was from BD Biosciences, anti-human CD3-FITC, CD19-PE, CD4-PE, and CD8-PE were from Beckman Coulter. For all inhibitors presented in our results, we used either previously reported concentrations [45] or extensive titrations were done before the final experiments.

Cell lines and viruses

Jurkat, Hut-78 (both T lymphocytic cell lines) and *Spodoptera frugiperda* Sf9 cells were purchased from ATCC. Jurkat and Hut-78 cells were grown in RPMI 1640 (Gibco), supplemented with 10% (v/v) FCS 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C/5% CO_2. Sf9 cells were maintained in SF900II SFM (Gibco) supplemented with 5% (v/v) FCS. The Sf9 cell line was used for the generation and propagation of recombinant baculoviruses Bac1432 [27] and Bac1746-control virus [26]. Briefly, the generation was performed according to standard protocols using Baculogold DNA (BD Biosciences), and propagation of viruses was performed in SF900 medium supplemented with 5% (v/v) FCS and 50 μg/ml of gentamicin (Gibco).

Peripheral blood mononuclear cells and intrahepatic mononuclear cells isolation

Peripheral blood was collected from healthy donors following consent at Hippokration Hospital of Athens. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque 1,077 g/l (Sigma Chemicals) density centrifugation at 1,700 rpm for 30 min at room temperature and the interface containing PBMCs was carefully aspirated. PBMCs were then placed in RPMI containing 10% FBS and washed at 2,000 rpm for 10 min. The pellet (PBMCs) was resuspended in RPMI 1640 containing 1% (v/v) FBS. The viability of PBMCs was estimated with 7-AAD (7-actinomycin-D) antibody (BD Biosciences) by flow cytometry (EPICS XL, Beckman Coulter).

The intrahepatic mononuclear cell population was obtained from human liver biopsy from a healthy donor following consent. Collection of the liver specimens was approved by the Ethical and Scientific Committee of Hippokration Hospital of Athens. The liver biopsy was treated with Collagenase Type IV (Gibco). Parenchymal cells were pelleted after centrifugation. The supernatant containing the non-parenchymal cells was centrifuged and resuspended in RPMI 1640. Then, the non-parenchymal cells were placed over Histopaque 1,077 g/l and centrifuged at 1,700 rpm for 30 min at room temperature. The interface containing the intrahepatic mononuclear cells was washed at 2,000 rpm for 10 min and resuspended in RPMI 1640 with 1% FBS. From the mononuclear cells, T lymphocytes were characterized with anti-human CD3-FITC antibody (Beckman Coulter). The viability of mononuclear cells was again estimated with 7-AAD antibody.

Production of HCV non-enveloped capsid-like particles

The capsid-like particles (HCVne) were isolated from cell lysates, as previously described [26, 27]. Fractions (650 μl each) were collected from the top of the gradient, the density was determined by refractometry, and HCV antigen was analyzed both with the ortho HCV core antigen ELISA test system (dilution 1:1,000 in PBS), and by SDS-PAGE followed by immunoblotting.

Immunofluorescence and flow cytometry

T cells (approximately 10^6 cells) grown in 12-well culture plates (Corning, NY, USA) were starved by incubation in RPMI without serum for 48 h. Following starvation, cells were incubated with HCVne, corresponding to 40 ng of core protein, for 40 min at 4°C and 60 min at 37°C. Following incubation, non-bound material was removed by three washes in PBS. Cells were fixed with 4% (w/v) paraformaldehyde in PBS (30 min) and the remaining reactive groups were blocked with 100 mM glycine. Cells were permeabilized with 0.1% saponin (Fluka) in PBS for 15 min and incubated with anti-core mAb (Alexis Biochemicals) at a dilution 1:100 for 60 min followed by incubation with anti-mouse Alexa Fluor 488 conjugated secondary antibody diluted 1:2,000 for 45 min. After washing three times with permeabilization buffer, cell pellets were resuspended in 500 μl of 2% FCS in PBS. Cell-bound fluorescence was analyzed with a FACS-calibur flow cytometer (Becton-Dickinson) by using Cellquest 3.11 software. All mean fluorescence intensity values were obtained using histogram statistic tools via this software. Using gating, we subtracted dead cells and cells that were non-specifically fluorescent. The mean fluorescence intensity directly relates to the bound/internalized HCVne and was determined after subtraction of non-specific fluorescence. Autofluorescence, as determined from mock-treated cells, was also subtracted. Non-specific fluorescence was determined by incubating the cells with equal volumes of heat denatured HCVne or core soluble protein.

For the characterization of CD4+, CD8+ T cells and CD19+ B cells by flow cytometry, human primary PBMCs (approximately 10^6 cells) grown in 12-well culture plates (Corning, NY, USA) were treated as previously described and incubated with HCVne, corresponding to 40 ng of core protein, for 40 min at 4°C and 60 min at 37°C. Following incubation, non-bound material was removed by three washes in PBS. PBMCs were stained with the surface antibodies CD4, CD8, and CD19 in order to characterize the T and B cell population. Cells were incubated separately with anti-human CD4-PE, CD8-PE, and CD19-PE antibodies for 20 min RT in the dark. The unbound antibodies were removed by centrifugation at 2,000 rpm for 10 min. Cells were fixed/permeabilized with 4% (w/v) paraformaldehyde in addition to 0.1% saponin in PBS (30 min) and core protein was intracellularly stained as previously described. Cells were immediately analyzed by EPICS XL (Beckman Coulter) flow cytometer.

Plasmids and transient transfections

The IL-2 promoter (−326 to +45) luciferase reporter construct IL-2/luc was a gift of Dr. M. Leonardo (National Institutes of Health, Bethesda, MD, USA) [39]. pEgr1.1-luc (−237 to +235) and pEgr1.2-luc (−492 to +235) plasmids were kindly provided by Dr. Gerald Thiel [46].

Jurkat cells were transfected by electroporation using a gene pulser (Gene Pulser^{TM} Transfection Apparatus, Bio-Rad) at 250 V/960 μF. For transfections with IL-2/luc, pEgr1.1/luc and pEgr1.2/luc, 10 μg of plasmid and 2×10^6 cells were used. Following transfection with IL-2/luc, cells were rested for 24 h and then stimulated with 2 ng/ml PMA and 200 ng/ml ionomycin for 4 h at 37°C. After stimulation, cells were either incubated with HCVne, corresponding to 80 ng of core protein, or with the negative controls, for different time points. Subsequently, cell extracts were prepared and luciferase assays were performed using the Luciferase Assay System (Promega), according to the manufacturer's instructions. Protein concentration was determined using the Bradford assay kit (Bio-Rad) and used for normalization of the luciferase values. All experiments were repeated five times.

Western blotting

Cells were lysed in ice-cold lysis buffer (1%(v/v) Triton X-100, 50 mM KCl, 10 mM Tris pH 7.5, 1 mM DTT, 2 mM $MgCl_2$, Complete-Mini Protease Inhibitor Cocktail Tablets (Roche), 1 mM PMSF, 2 mM sodium orthovanadate). Protein concentration in each sample was measured and equally loaded. Samples were electrophoretically separated on 10–12% (w/v) SDS gels, transferred onto nitrocellulose membranes, incubated with the appropriate antibodies, and detected by enhanced chemiluminescence (Pierce). For the detection of phopsho-CREB and c-fos, nuclear extracts were used. Preparation of nuclear and cytoplasmic extracts has been described elsewhere [47]. The program Quantity One 4.4.1 (Bio-Rad) was used for the densitometric analysis of gels.

mRNA expression analysis

Jurkat or Hut-78 cells (approximately 10^6 cells) grown in 12-well culture plates were starved by incubation in RPMI without serum for 48 h. Following starvation, cells were incubated with HCVne, corresponding to 80 ng of core protein, or heat denatured controls, for different time points. Following incubation, total cellular RNA was extracted using the Nucleospin RNA II-Total RNA Isolation kit (Macherey-Nagel) or TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration, purity, and integrity of RNA samples were assessed by A260/280 spectrophotometric measurement and gel analysis. RNA (1 μg) was reversely transcribed using MMLV reverse transcriptase (Promega) and the resulting

cDNA was subjected to semi-quantitative PCR analysis. The following primers were used: IL-2 [39], *c-fos* [21], *egr-1* [48], IFN-γ [49] and 28S rRNA (as an internal PCR control) [50]. All PCR conditions were designed to be in the exponential phase of amplification and, therefore, provided a direct correlation between the amount of products and RNA template abundance in the samples. The PCR products were analyzed on a 2% (w/v) agarose gel and the Quantity-One 4.4.1 software (BioRad) was employed for densitometric analysis of the gels.

IL-2 ELISA

Hut-78 or Jurkat cells (10^6 cells/sample) grown in 12-well culture plates were starved and incubated with HCVne, as described above. The levels of secreted IL-2 were determined by human IL-2 ELISA (Bender MedSystems GmBH) using 100 µl of the cell culture supernatant and a pair of unlabeled and biotin-labeled, IL-2-specific monoclonal antibodies, according to the manufacturer's instructions. All measurements were conducted in duplicate cultures.

Statistical analysis

Statistical analysis of significance between control and treated samples from the PCR quantifications are expressed as means ± standard deviation and were performed using Student's t test. The level of significance was set as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Binding and uptake of non-enveloped particles (HCVne) by T lymphocytes

Jurkat cells or Hut78 (data not shown) were incubated with HCVne at 4°C for 40 min and then subjected to core-specific monoclonal antibody surface staining followed by FACS analysis. In this assay, positive fluorescence shifts represented specific core–cell surface interactions. As shown in Fig. 1Aa, HCVne bind to T cells in a dose-dependent manner, since the levels of cell-bound particles are increased from 17.1 to 33%, depending on HCVne core concentration. No shift was observed with identical samples of cells that had been incubated with the corresponding fraction from the control virus Bac 1746 (Fig. 1Ab) that expresses GFP instead of core (described in [26]), or heat-denatured HCVne (95°C for 40 min) (Fig. 1Ac). An additional control included cells that had been treated with purified bacterial soluble core protein with the same concentration (Fig. 1Ad).

In order to achieve cellular uptake from the largest possible number of cells, cell–HCVne complexes must be formed at 4°C and then moved to a 37°C environment [51]. This technique was utilized to test whether T lymphocytes were permissive to HCVne uptake. Intracellular staining of the internalized HCVne by core antibody and FACS analysis was performed immediately after the removal of the inoculum. As shown in Fig. 1Ba, 34–43% of Jurkat cells were detected as core-positive in a dose-dependent manner. Cells that had been incubated with the corresponding fraction from the control virus Bac 1746 (Fig. 1Bb), or with heat-denatured HCVne (Fig. 1Bc), or with purified bacterial soluble core protein with the same concentration (Fig. 1Bd), were used as controls. Nevertheless, the observed signal was very low, indicating that only HCVne enter the cells efficiently. Furthermore, Jurkat cells that had been incubated with the HCVne were subjected to proteinase K (50 µg/ml) treatment at different incubation time points, at 37°C. After digestion of bound particles with proteinase K for 20 min at 4°C, immunoreactive core signal was clearly detectable in the cells (Fig. S1, supplementary material). Conversely, when incubation was at 4°C, binding of HCVne did occur, but no intracellular signal was recorded (data not shown). Similar results concerning binding and uptake of particles by different types of immune cells were obtained with FACS analysis, using green fluorescent GFP-tagged non-enveloped particles instead [26].

In order to verify the specificity of these events, we tested the inhibitory effect of core-specific antibody to HCVne uptake. To assess the inhibition of HCVne uptake by Jurkat cells, the capsids were pre-incubated for 1 h at 37°C with polyclonal core antibody at different dilutions. Then, HCVne–antibody complexes were added to the cell culture and incubated for 1 h at 37°C. The uptake of HCVne by Jurkat cells was determined by intracellular core detection, as described above and HCVne uptake was found to be inhibited in a dose-dependent way (Fig. 1Ca). In contrast, when pre-treatment with different dilutions of GFP polyclonal antibody was carried out, there was no inhibition of HCVne uptake (Fig. 1Cb).

HCVne particles mediate activation of MAPKs: p38 and ERKs

Next, we examined whether the binding/uptake of HCVne leads to T cell activation. Several lines of evidence indicate that signals emanating from TCR or other costimulatory receptors converge on MAPKs. Activated MAPKs, phosphorylate and activate various downstream molecules, resulting in T cell activation and proliferation [52]. Hut-78 cells (5×10^5 cells/sample) were starved for 48 h and subsequently challenged with HCVne (approximately

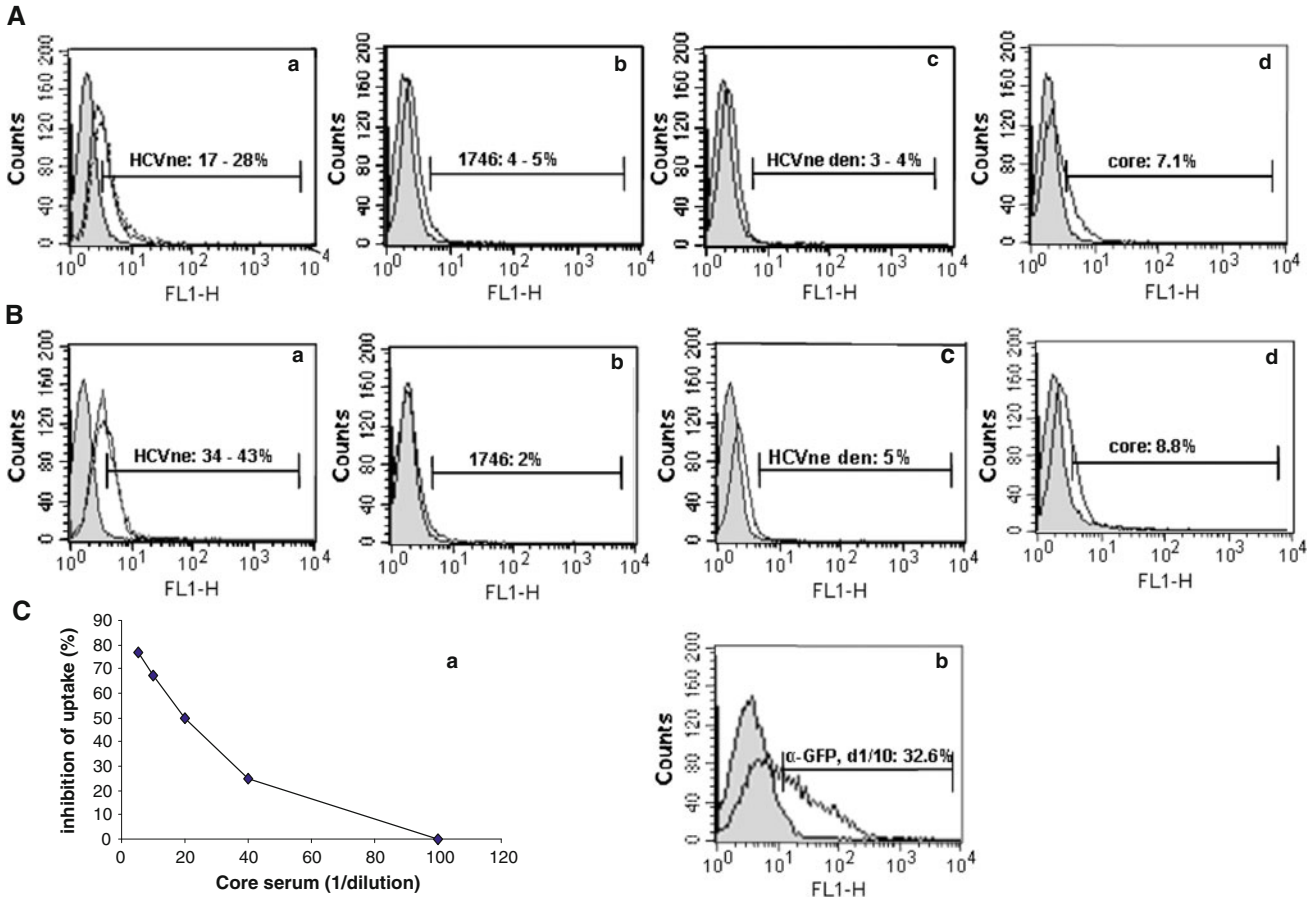


Fig. 1 HCVne particles enter T lymphocytes. **A** FACS analysis of surface immumolabeled Jurkat cells maintained in serum-free conditions incubated with: *a* increasing concentrations of HCVne (20 ng and 40 ng of core protein, represented by *continuous* and *dotted line*, respectively) or treated with: *b* a fraction of equivalent sucrose density from Sf9 cell lysates infected with the control baculovirus (Bac1746) or *c* the same concentrations of the HCVne fraction heat-denatured (40 min at 95°C), for 40 min at 4°C. An additional control *d* includes cells treated with the same concentration of purified bacterial soluble core protein (40 ng). **B** FACS analysis of identical samples further incubated for 60 min at 37°C and intracellularly stained with core antibody. **C** Concentration-dependent anti-core antibody mediated inhibition of cellular HCVne uptake. HCVne particles were incubated with various dilutions of core polyclonal antibody as indicated on the *x*-axis. HCVne-antibody complexes were added to Jurkat cells for 1 h at 37°C and cells were subjected to intracellular core immunostaining with a monoclonal antibody against the core antigen (*a*). FACS analysis of Jurkat cells that were incubated with HCVne pre-treated with a GFP polyclonal (as a non-core control antibody) antibody (dilution 1/10) and subjected to intracellular core immunostaining (*b*). In all histogram plots, the *grey-shaded curve* represents mock-treated cells

20 ng core) for various time points. As shown in Fig. 2A, at 30-min post-incubation at 37°C, an approximate 1.7-fold activation of p38 was observed. This induction remained stable for up to 12 h, whereby it was increased up to threefold. After 12 h, the activation signal was gradually decreased (data not shown). Incubation with 10 μM of anisomycin, a well-known stimulator of the pathway, for 1 h was performed as a positive control. In addition, cells incubated with a corresponding fraction from the control virus Bac1746 expressing GFP protein instead of core, were used as negative control. MAPK-p38 activation correlated positively to increasing HCVne particles concentrations (Fig. 2Ba, b, c). When identical samples treated with heat-denatured HCVne were used, no p38 activation was observed. Similar results concerning p38 activation were also obtained with Jurkat cells (Fig. S2, supplementary material).

It has been reported that activated p38 translocates to the cell nucleus, phosphorylates CREB via mitogen - and stress- kinase 1 and 2 (MSK 1/2). This event stimulates CREB-mediated transcription of several genes [53]. To investigate whether p38 activation by HCVne also induces these events, Hut-78 cells were starved for 48 h and challenged with HCVne particles at various time points, as already described. Figure 2Ca, b shows that 30-min post-incubation at 37°C, an approximate threefold phosphorylation of CREB, was observed in the nucleus. This was sustained for up to 12 h post-incubation (p.i.). The CREB-related protein, ATF-1, followed the same phosphorylation pattern (Fig. 2Cc, d). As a positive control for CREB

phosphorylation, cells were incubated with 10 μM forskolin for 1 h. The same results were obtained with Jurkat cells (data not shown). Overall, the above results suggest a possible role for the p38 signaling cascade being activated when T cells are incubated with HCVne.

In parallel experiments, we examined the activity of MAPK-ERK 1/2 in T cells challenged with HCVne particles. MAPK-$ERK_{1/2}$ pathway is involved in the control of many fundamental cellular processes and is targeted by different viruses [54, 55]. Furthermore, previous results from our laboratory have already shown an HCVne-dependent activation of ERK1/2, in cells of hepatic origin [26, 27]. Hut-78 cells were challenged with HCVne at different time points as already described. We observed that 1 h post-incubation at 37°C, an approximate twofold activation of ERK1 and a 2.5-fold activation of ERK2 were seen. Again, the activation was sustained for up to 6 h and decreased gradually after 12 h post-incubation. As a positive control for ERK1/2 activation, cells were treated with 40% FCS for 15 min. Additionally, no ERK1/2 activation was observed in cells incubated with a control fraction from Bac 1746 (Fig. 2Da, b, c). Similar results were obtained with Jurkat cells (data not shown). In view of these results, we propose that incubation of T cells with HCVne leads to a parallel activation of the p38 and ERK1/2 signaling pathways.

Uptake of HCVne particles leads to transcriptional activation of IL-2

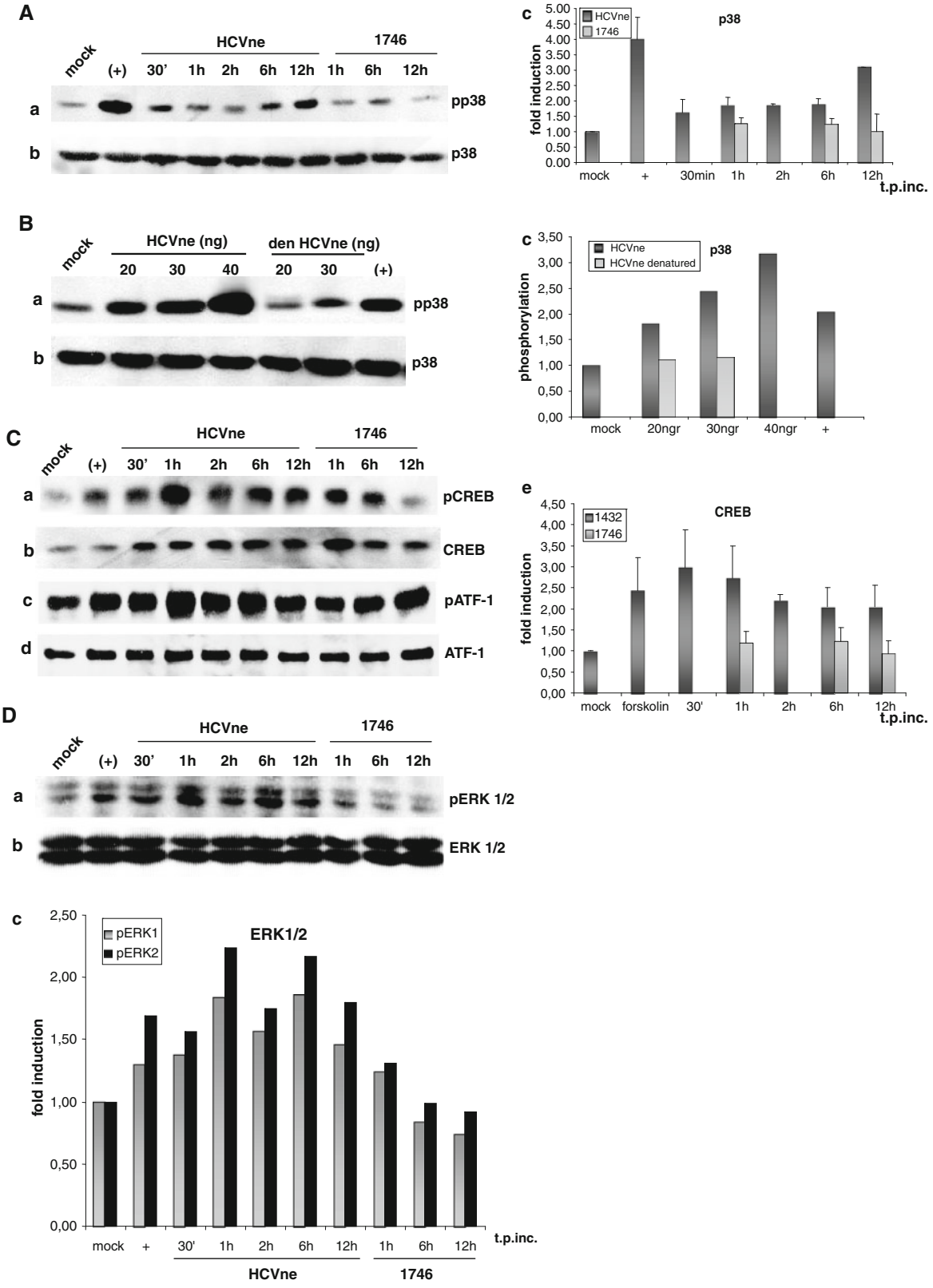
IL-2 is an inflammatory cytokine produced primarily by helper T cells and has a central role in the regulation of host responses to pathogenic challenges. Its expression is mainly transcriptionally regulated. It is accepted that IL-2 mRNA production and expression can be detected only following cell stimulation with PMA/ionomycin in Jurkat T cells [56, 57]. To investigate whether the IL-2 promoter could be activated upon HCVne binding/uptake, Jurkat cells (2×10^6 cells/sample) were electroporated with the (−326/+45) IL-2 promoter reporter construct. The cells were then stimulated 36 h later with PMA/ionomycin for 4 h and challenged with HCVne at various time points. For this series of experiments, we used Jurkat cells, because they are relatively easy to transfect by electroporation. Indeed, its transfectability was higher than that of Hut-78 cells, and almost reached 70%. As shown in Fig. 3A, a significant activation of 2–2.5-fold was observed during the first 6 h post-incubation, as compared to the PMA-stimulated control, whereupon the activity of the promoter dropped for the duration of treatment. No induction was detected in cells incubated with the corresponding control fraction from Bac 1746.

To further investigate the possibility of IL-2 modulation, we examined IL-2 transcriptional activation by checking endogenous mRNA levels of this gene, in T cells after HCVne binding and uptake, normalized against 28S RNA. For this purpose, Hut-78 cells (8×10^5 cells/sample) were starved for 48 h and were subsequently challenged with HCVne (approximately 20 ng core) in various time points. Cells stimulated with PMA/ionomycin for 4 h served as positive control for the experiment. IL-2 mRNA was up-regulated starting from 1 h post-incubation and remains elevated up to 12 h post-incubation of the T cells with the HCVne, in agreement with the IL-2 gene reporter assay (Fig. 3B). After 12 h, mRNA levels were decreased and gradually drop to basal (mock) levels at 48 h post-incubation (data not shown). The same HCVne fraction but heat denatured showed no induction of IL-2 mRNA. Similar results were obtained in Jurkat cells pre-stimulated with PMA/ionomycin where IL-2 was further upregulated in the samples that were incubated with HCVne for different time points and dropped to basal levels at 72 h post-incubation (S3A, supplementary material). Taken together, these results suggest that the binding and subsequent uptake of HCVne particles by T cells leads to a transient transcriptional activation of IL-2 thus, potentially triggering host immune response.

Induction of immediate early genes (IEG) that regulate IL-2 transcription

The induction of IEG following exposure to extracellular stimuli represents the first major transcriptional program that precedes changes in a variety of cellular responses. The early growth response-1 gene (*egr-1*) is induced by a wide range of stimuli in diverse cell types including T cells. Egr-1 is an important activator of the IL-2 gene. *egr-1* promoter contains a CRE *cis*-acting element where phosphorylated forms of CREB and ATF-1 bind and induce its expression. Their phosphorylation is p38-dependent [58].

To investigate whether HCVne particles binding/uptake could affect the transcription of *egr-1*, Jurkat cells (2×10^6 cells/sample) were electroporated with the *egr-1.2/luc* plasmid, for 36 h and subsequently challenged with HCVne at different time points. As shown in Fig. 4Aa, luciferase activity is highly up-regulated (sixfold) at 2 h post-incubation and remains elevated until 24 h post-incubation compared to mock-treated cells. Cells stimulated with PMA/ionomycin served as positive control for the experiment. Cells incubated with the corresponding control fraction from Bac 1746, showed relatively low activation levels. It is of note that when a similar experiment was performed by using the truncated *egr-1* reporter (*egr-1.1/luc)* plasmid (where *o*nly CRE and two SREs are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a
pCREB
b
CREB
c
pATF-1
d
ATF-1
e
1432
1746
CREB
fold induction
mock
forskolin
30'
1h
2h
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12h
t.p.inc.
D
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30'
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1746
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pERK 1/2
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pERK1
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fold induction
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HCVne
1746

◀ **Fig. 2** HCVne particles mediate activation of MAPKs. **A** Time course of p38 phosphorylation from serum-starved Hut-78 cells incubated with HCVne particles (15 ng of core protein) for 30 min, 1, 2, 6, and 12 h at 37°C. Anisomycin-treated cells (10 μM) were used as the positive control. Cells treated with a fraction of equivalent sucrose density from Sf9 cell lysates infected with Bac1746 were used as negative controls. Western blot (one representative experiment out of three) with phospho-p38 (*a*), p38 (*b*), and quantification of the optical densities of phospho-p38 immunoreactive bands normalized to the optical densities of total p38 in the same samples (*c*) are presented. **B** Serum-starved Hut-78 cells were incubated with increasing concentrations of HCVne particles (expressed in ng of core protein). The same positive and negative controls were used as in **A**. Western blot with phospho-p38 (*a*), p38 (*b*), and their quantification performed as described earlier (*c*) are presented. **C** Time course of CREB and CREB-related protein ATF-1 phosphorylation from nuclear extracts of serum-starved Hut-78 cells incubated with HCVne for 30 min, 1, 2, 6 and 12 h at 37°C. Forskolin (10 μM) was used as positive control. Negative controls are already described in **A**. Western blot (representative experiment of triplicates) with phospho-pCREB (*a*), CREB (*b*), phospho-ATF-1 (*c*), ATF-1 (*d*), and quantification analysis after normalization of pCREB (*e*) are presented. In panels **A**, **B** and **C**, samples treated with HCVne and positive control are represented in *dark grey bars* and negative controls are represented in *light grey bars*. **D** Time course of $ERK_{1/2}$ phosphorylation from serum-starved Hut-78 cell lysates incubated with HCVne particles for 30 min, 1, 2, 6 and 12 h at 37°C. Cells treated with 40% FCS were used as positive control. Negative controls are already described in **A**. Western blot with phospho-$ERK_{1/2}$ (*a*), $ERK_{1/2}$ (*b*), and quantification analysis after normalization (*c*) are presented. pERK1 is represented in *light grey bars* and pERK2 in *dark grey bars*

present) [46], the level of activation was greatly down-regulated (Fig. 4Ab). This means that responsive elements between −492 and −237 are essential for the HCVne transcriptional activation of *egr-1* that will subsequently stimulate IL-2 transcription further verifying our hypothesis that p38 signaling pathway participated in IL-2 regulation.

Next, the endogenous mRNA levels of *egr-1* gene in Hut-78 cells were tested (Fig. 4Ba, b). Upregulation was observed from 30 min reaching the maximum at 2 h post-incubation with HCVne particles as compared to the negative control samples.

c-fos, represents another IL-2 regulating IEG. It is known that *c-fos* and *c-Jun* homo- or hetero-dimerize to form the AP-1 transcription factor which binds to the IL-2 enhancer and activates its expression. *c-fos* expression is mainly regulated by ERK 1/2 [59]. Endogenous mRNA levels of *c-fos* gene in Hut-78 cells (Fig. 4Ca, b) were found to be upregulated from 30 min reaching a maximum at 1 h post-incubation with HCVne particles. In addition, high steady-state levels of c-Fos protein in nuclear extracts of cells incubated with HCVne particles until late time points (Fig. 4Cc) indicated high protein stability. This result is in agreement with the almost sustained $ERK_{1/2}$ activation already observed in Fig. 2D.

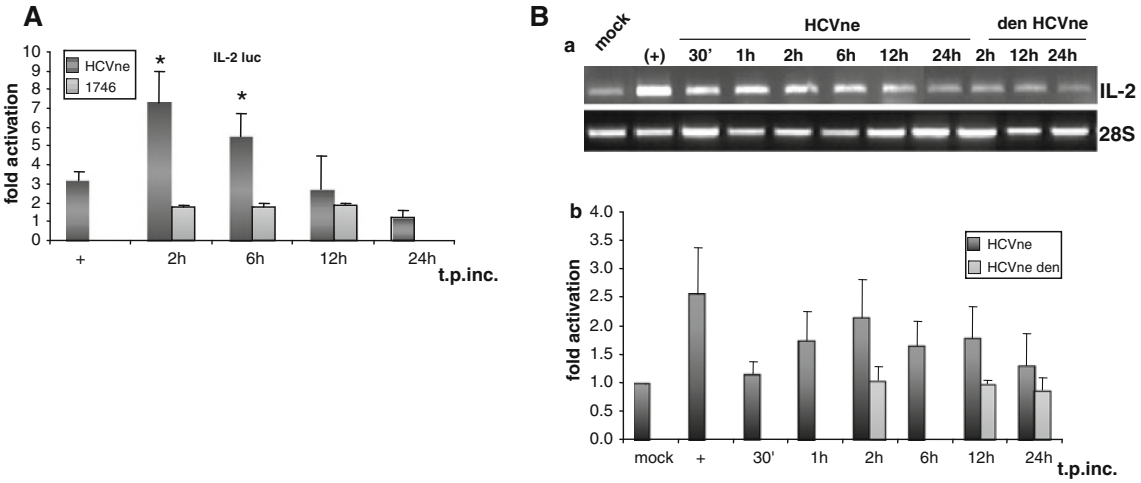

Fig. 3 Uptake of HCVne particles leads to transcriptional activation of IL-2. **A** Jurkat cells were transfected with *IL-2/luc* plasmid. After 36 h, the cells were stimulated with PMA/ionomycin for 4 h and subsequently challenged with HCVne for 2, 6, 12, and 24 h at 37°C. Cells treated with a fraction of equivalent sucrose density from Sf9 cell lysates infected with Bac1746 were used as negative controls. Luciferase values were normalized to total protein. **B** Total mRNA was isolated from serum-starved Hut-78 cells that were incubated with HCVne particles for 30 min, 1, 2, 6, 12, and 24 h at 37°C and were subjected to RT-PCR. Cells stimulated with PMA/ionomycin were used as a positive control for the experiment. As negative controls, heat-denatured HCVne (95°C for 40 min) were used. PCR was performed with specific primers for *IL-2* and *28S RNA* (*a*). The same experiment was repeated five times and a representative image is presented. Densitometric analysis after normalization is presented in arbitrary units (*b*). Samples treated with HCVne and positive control are represented in *dark grey bars* and negative controls are represented in *light grey bars*

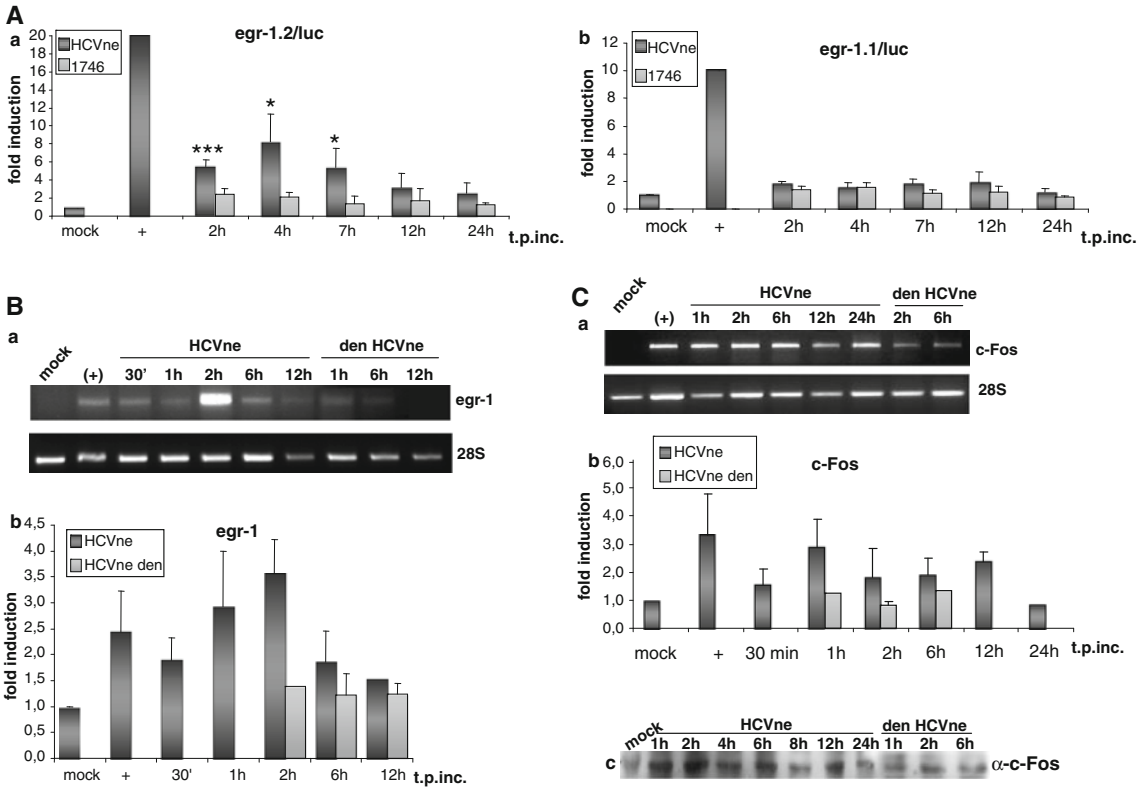

Fig. 4 Uptake of HCVne particles induces IEGs that regulate IL-2 transcription. **A** Jurkat cells were transfected with *egr-1.2/luc* plasmid and 36 h later challenged with HCVne for 2, 4, 7, 12, and 24 h at 37°C. Cells treated with a fraction of equivalent sucrose density from Sf9 cell lysates infected with Bac1746 were used as negative controls. Cells stimulated with PMA/ionomycin for 4 h were used as a positive control. Luciferase values were normalized to total protein (*a*). The same experimental procedures were performed following transfection of Jurkat cells with *egr-1.1/luc* plasmid, instead of *egr-1.2/luc* plasmid (*b*). All measurements were carried out in triplicates. **B** Total mRNAs were isolated from serum-starved Hut-78 cells that were incubated with HCVne for 30 min, 1, 2, 6, and 12 h at 37°C and were subjected to RT-PCR. The positive control used is described in **A**. As negative controls, heat-denatured HCVne (95°C for 40 min) were used in selected time points. PCR was performed with specific primers for *egr-1* and 28S (*a*) (one representative experiment out of three). Densitometric results were normalized against 28S RNA and are presented in arbitrary units (*b*). **C** Total mRNAs were isolated from serum-starved Hut-78 cells that were incubated with HCVne for 1, 2, 6, 12, and 24 h at 37°C and were subjected to RT-PCR. The positive and negative controls are described in **B**. PCR was performed with specific primers for *c-fos* and *28S RNA* (*a*) (representative experiment of triplicates). Densitometric results were normalized against 28S and are presented in arbitrary units (*b*). Nuclear extracts of serum-starved Hut-78 cells treated with HCVne for 1, 2, 4, 6, 8, 12, and 24 h at 37°C immunostained with c-fos antibody (*c*). As negative controls, nuclear extracts from cells treated with heat-denatured HCVne (95°C for 40 min) were used in selected time points. In all panels, samples treated with HCVne and positive control are represented in *dark grey bars* and negative controls are represented in *light grey bars*

In summary, transcriptional induction of both *egr-1* and *c-fos* genes, although slightly reduced after 2 h, remained evident for up to 12 h following exposure of cells to HCVne particles.

Transcriptional activation of IL-2 is mediated by p38 phosphorylation

In light of previous reports demonstrating that IL-2 expression is regulated at the transcriptional level and that several transcription factors of IL-2 promoter are regulated by MAPK-p38, experiments were designed to determine whether the uptake of HCVne particles leads to transcriptional activation of IL-2 via p38 phosphorylation. It is of note that numerous reports demonstrate that p38 signaling pathway regulates directly or indirectly IL-2 transcription with contrasting results [40].

Thus, we examined the influence of p38 inhibition to IL-2 transcriptional activation upon HCVne particles uptake. After electroporation of Jurkat T cells with the *IL-2/luc* reporter plasmid, all samples were stimulated with PMA/ionomycin for 4 h. Subsequently, PMA and ionomycin were removed and 2.5 μM of SB203580, a widely used p38 inhibitor, was added in order to block p38 phosphorylation completely (data not shown). Twelve hours later, HCVne particles were added for various times of incubation. As shown in Fig. 5A, the activation level of IL-2 promoter in all samples is approximately the same as the stimulated

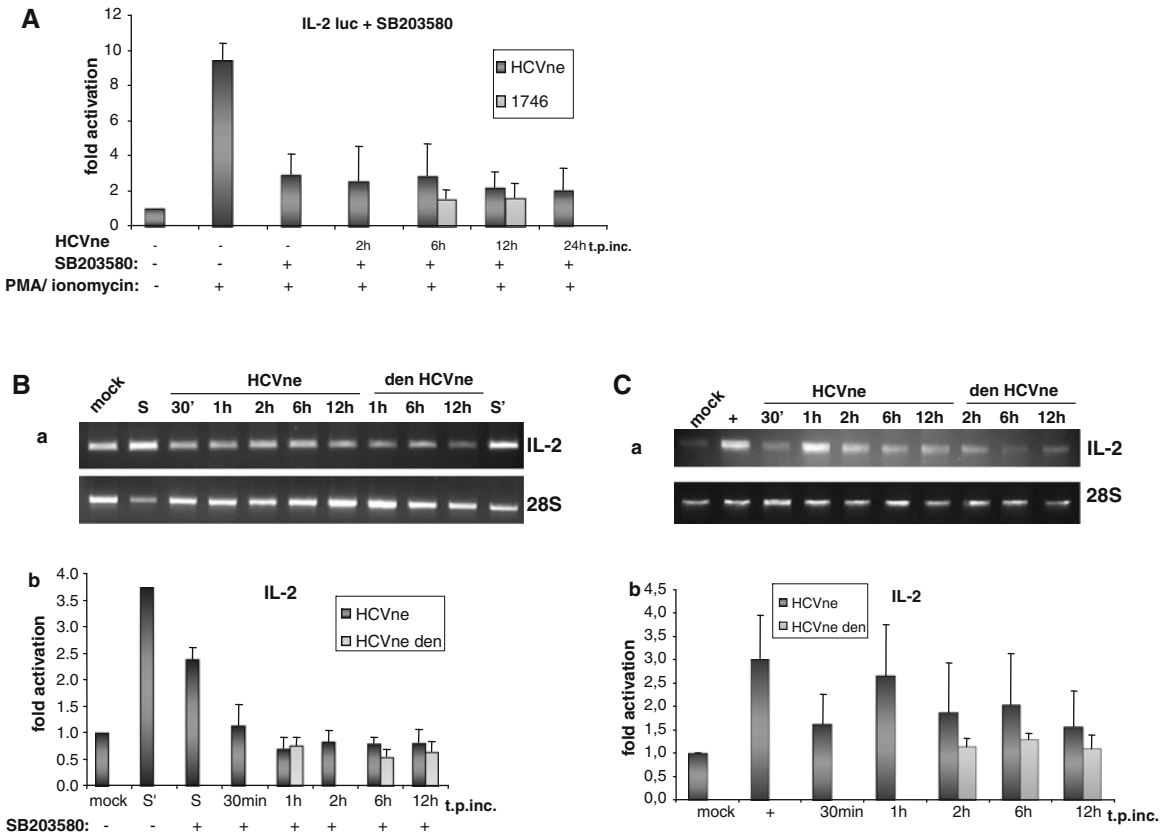

Fig. 5 Transcriptional activation of IL-2 is mediated by p38 phosphorylation. **A** Jurkat cells were transfected with *IL-2/luc* plasmid. After 36 h, the cells were stimulated with PMA/ionomycin for 4 h, treated with 2.5 μM of SB203580 for 12 h, and subsequently challenged with HCVne for 2, 6, 12, and 24 h at 37°C. Cells treated with the corresponding fraction of the control virus Bac1746 were used as negative controls. Luciferase values were normalized to total protein. **B** Total mRNAs were isolated from serum-starved Hut-78 cells that were treated with 2.5 μM of SB203580 for 12 h and subsequently incubated with HCVne particles for 30 min, 1, 2, 6, and 12 h at 37°C, and were subjected to RT-PCR. Cells stimulated with PMA/ionomycin for 4 h with (S) or without SB203580 (S′) pretreatment were used as positive control. As negative controls, heat-denatured HCVne (95°C for 40 min) were used. PCR was performed with specific primers for *IL-2* and *28S RNA* (*a*) (one representative experiment out of three). Densitometric analysis after normalization is presented in arbitrary units (*b*). **C** An identical experimental procedure was performed using 2 μM UO126 for 12 h, instead of SB203580. *IL-2* and *28S RNA* (*a*) PCRs are presented (one representative experiment out of three) as well as densitometric analysis after normalization in arbitrary units (*b*). Cells stimulated with PMA/ionomycin for 4 h in the presence of UO126 were used as a positive control. In all panels, samples treated with HCVne and positive control are represented in *dark grey bars* and negative controls are represented in *light grey bars*

sample (basal levels), suggesting that the IL-2 promoter could not be further activated upon HCVne particle uptake. This lack of further activation provided evidence that the subsequent p38 inhibition was capable of blocking IL-2 transcription.

This effect was further verified by investigating IL-2 mRNA levels after p38 inhibition. For this purpose, Hut-78 T cells (8×10^5 cells/sample) were starved for 48 h, treated with 2.5 μM SB203580 for 12 h, and were subsequently challenged with HCVne, as already described. As a positive control, cells were stimulated with PMA/ionomycin for 4 h (Fig. 5Ba). As shown in Fig. 5Ba, IL-2 mRNA levels were similar to the levels of mock-untreated cells. The corresponding graph (Fig. 5Bb) showed no increase in the IL-2 mRNA levels after HCVne particles treatment at various times post-incubation. The same procedure but with heat-denatured HCVne fraction showed no activation.

Taking into consideration the above results, as well as the comparison of IL-2 transcriptional activation between treated and non-treated with SB203580 samples (Fig. S3B, supplementary material), we conclude that IL-2 transcription is blocked when p38 is not activated.

Furthermore, we used a MEK inhibitor in order to investigate the role of $ERK_{1/2}$ on IL-2 transcriptional activation. For this purpose, Hut-78 cells (8×10^5 cells/sample) were starved for 48 h, treated with 2 μM UO126 for 12 h, and were subsequently challenged with HCVne particles as already described. UO126 is an established specific MEK inhibitor that blocks ERK activation.

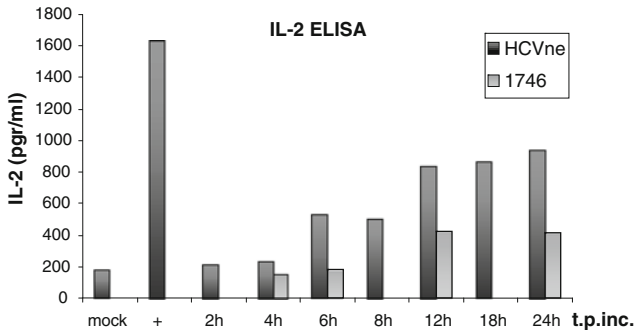

Fig. 6 Uptake of HCVne particles leads to IL-2 secretion. Serum-starved Hut-78 cells challenged with HCVne for 2, 4, 6, 8, 12, and 24 h at 37°C and IL-2 ELISA was performed in the culture supernatants. The levels of secreted IL-2 were measured in pg/ml. As a positive control, cells were stimulated with PMA/ionomycin for 4 h. Supernatants from cells incubated with a corresponding fraction from Bac 1746 were used as negative controls. Samples treated with HCVne and positive control are represented in *dark grey bars*, while negative controls are represented in *light grey bars*. All ELISA tests were performed in duplicate and repeated twice

Results, obtained after RT-PCR and IL-2 PCR, indicate that ERK 1/2 inhibition has no significant effect on IL-2 transcriptional activation, since IL-2 mRNA levels were elevated from 30 min up to 12 h post-incubation with HCVne (Fig. 5C). Data obtained from cells treated with heat-denatured HCVne showed no IL-2 mRNA increase. In conclusion, these results provide evidence that HCVne particles induce IL-2 transcription via the p38 signaling cascade.

Uptake of HCVne particles leads to IL-2 secretion

In order to investigate IL-2 secretion after incubation of T cells with HCVne particles, ELISA assays were performed in the supernatants from Hut-78 cells (8×10^5 cells/sample) starved and incubated with HCVne, as already described. Figure 6 demonstrates a gradual rise in IL-2 protein levels, starting at 6 h post-incubation of T cells with HCVne with a pick at 24 h corresponding to a four-fold increase in comparison to mock-treated cells. Cells stimulated with PMA/ionomycin for 4 h exhibited a nine-fold activation. On the contrary, cells incubated with a corresponding fraction from Bac 1746, showed a non-specific minimal stimulation, which is commonly observed in T cells [60]. Similar results were obtained in Jurkat cells pre-stimulated with PMA/ionomycin, as already described (supplementary S3C).

IFN-γ transcriptional activation in T cells treated with HCVne

It has been previously reported that endogenous IL-2 contributes to T cell expansion and IFN-γ production [33].

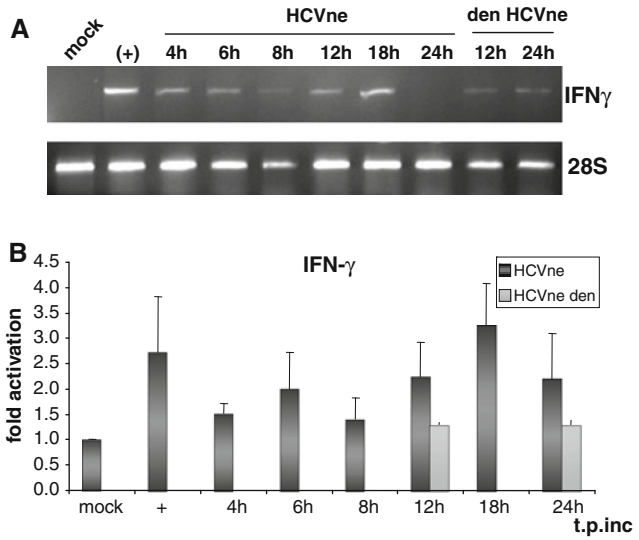

Fig. 7 IFN-γ transcriptional activation in T cells treated with HCVne. Total mRNAs were isolated from serum starved Hut-78 cells that were incubated with HCVne particles for 4, 6, 8, 12, 18, and 24 h at 37°C, and were subjected to RT-PCR. Cells stimulated with PMA/ionomycin for 4 h were used as a positive control. As negative controls, heat-denatured HCVne (95°C for 40 min) were used. PCR was performed with specific primers for *IFN-γ* and *28S RNA* (*a*). The same experiment was repeated three times and a representative experiment is presented. Densitometric analysis after normalization is presented in arbitrary units (*b*). Samples treated with HCVne and positive control are represented in *dark grey bars* and negative controls are represented in *light grey bars*

IFN-γ is a major product of Th1 cells and skews the immune response toward a Th1 phenotype. IFN-γ production is induced by several virus-like particles and is mainly controlled by cytokines secreted by antigen presenting cells, most notably IL-12 and IL-18 [61].

Following incubation of serum-starved Hut-78 cells with HCVne for various time points, an important increase in IFN-γ mRNA levels was observed (Fig. 7) compared to mock-treated cells. Cells stimulated with PMA/ionomycin for 4 h were used as positive control of IFN-γ production, whereas cells treated with a heat-denatured HCVne fraction provided no significant induction of IFN-γ mRNA. Consequently, the observed IFN-γ and IL-2 induction imply that the HCVne particles seem to have the potential to drive T cells towards a Th1-like response.

Uptake of HCVne particles by primary human T cells

Finally, we investigated the potential of HCVne internalization in primary PBMCs (5×10^5 cells/sample) isolated from a healthy donor. The uptake of HCVne by CD8+, CD4+ T cells (Fig. 8A) and CD19+ B cells (Fig. 8B) was determined by intracellular core detection in permeabilized cells by flow cytometry as previously described. The percentage ratio of core-positive cells was different in each

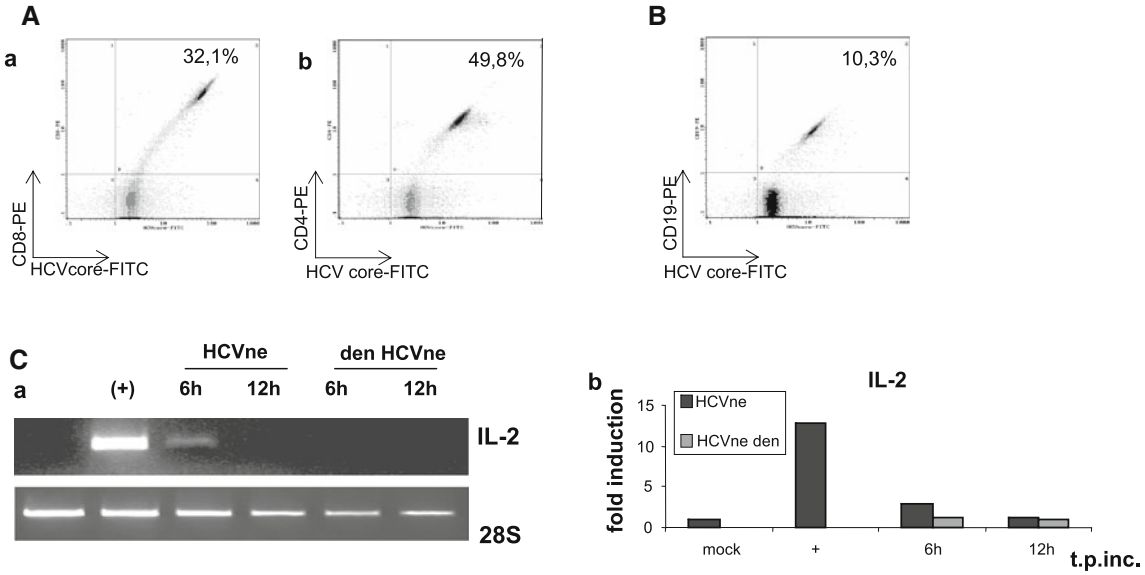

Fig. 8 Uptake of HCVne particles by primary PBMCs and IL-2 transcriptional activation. PBMCs (5×10^5 cells/sample) were incubated with HCVne (20 ng of core protein) at 4°C for 40 min and 1 h at 37°C. **A** Flow cytometry characterization of HCVne uptake in permeabilized cells. Representative plots of double staining of surface CD8+ T cells (*a*), CD4+ T cells (*b*) and intracellular detection of core protein. **B** Representative plot of double staining of surface CD19+ B cells and intracellular core protein. **C** Total mRNAs were isolated from primary human T cells that were incubated with HCVne particles for 6 h and 12 h at 37°C, and were subjected to RT-PCR. Cells stimulated with PMA/ionomycin for 4 h were used as experimental positive control. As negative controls, heat-denatured HCVne (95°C for 40 min) were used. PCR was performed with specific primers for *IL-2* and *28S RNA* (*a*). Densitometric analysis after normalization is presented in arbitrary units (*b*). Samples treated with HCVne and positive control are represented in *dark grey bars* and negative controls are represented in *light grey bars*

subset of PBMCs, while the total non-specific binding was 2.46% (data not shown), indicating that primary human PBMCs can efficiently internalize the HCVne particles. Representative plots of each subset indicate that CD8+ T cells (32.1%), CD4+ T cells (49.8%), and CD19+ B cells (10.3%) (Fig. 8Aa, b and Fig. 8B, respectively) were stained positive for core in respect to the unstained.

Additionally, primary human intrahepatic mononuclear cells, isolated from the liver biopsy of a healthy donor, were also permissive to HCVne uptake when the same experimental procedure was followed using HCVne and heat-denatured HCVne. According to preliminary data shown in Fig. S4a (supplementary material), 32% of intrahepatic mononuclears were detected as core-positive while the control sample (Fig. S4b) showed no significant intracellular signal (4.5%).

Moreover, binding or internalization of HCVne by primary PBMCs triggered IL-2 transcriptional activation, as demonstrated by the endogenous IL-2 mRNA levels (Fig. 8Ca). Specifically, PBMCs isolated from a healthy donor (3×10^5 cells/sample) were starved in RPMI with 1% (v/v) FCS for 12 h and were subsequently challenged with HCVne (approximately 20 ng core) at various time points or with PMA/ionomycin for 4 h. IL-2 mRNA was up-regulated (3.5-fold) at 6 h post-incubation and dropped back to basal levels of mock-treated cells at 12 h post-incubation (Fig. 8Cb). This was not seen with a heat-denatured HCVne fraction.

Overall, these results clearly demonstrate, for the first time, that HCVne particles can enter primary human PBMCs (CD4+ and CD8+ T cells) and intrahepatic mononuclear cells and this internalization is responsible for the transcriptional activation of IL-2.

Discussion

Modulation of signaling pathways by viruses is recognized as a key pathogenic determinant in viral diseases mediated by aberrant host immunological responses [62]. p38/MAPK activation has been documented at the early stages of infection with rhinoviruses, herpes viruses, HIV, SIV, and adenoviruses [54]. It is well established that the p38 MAPK pathway is responsible for the phosphorylation of a large group of transcriptional and translational response elements which directly regulate the expression of a wide variety of pro-inflammatory cytokines [63]. All MAPK cascades play an important role in regulating IL-2 expression. For example, activation of ERKs in T cells positively regulates IL-2 through AP-1. In contrast, as far as the p38 cascade is concerned, its effect on IL-2 regulation has not yet been clarified [53]. Inhibition of the p38/

MAPK signaling cascade down-regulates IL-2 promoter activity and IL-2 production in Jurkat T cells [64]. In primary T cells, however, although inhibition of p38/MAPK modulated IL-2 promoter activity, not only did it not reduce its expression, but in some cases it may even have increased it [53, 65]. Interestingly, the inhibitory effect of p38/MAPK on IL-2 expression could be shown to be a consequence of the inhibition of ERK activity [66].

The presence of HCV non-enveloped nucleocapsids (HCVne) in the serum of infected patients has been previously described [67], however, their role in HCV infection remains unclear. The detection of circulating, envelope-free HCV nucleocapsids in the serum may play a key role in establishing HCV infection and persistence. In our study, it was shown that HCVne can indeed regulate specific T cell signaling pathways involved in IL-2 and IFN-γ gene regulation, thus triggering host immune responses.

We also provide evidence that HCVne have the ability to bind and enter Jurkat T cells adequately, as well as human primary PBMCs and intrahepatic mononuclear cells. There is growing evidence that HCV virions have the ability to infect immune cells [17, 18, 68]. Internalization of HCVne particles has been previously described for human cells of hepatic origin and cells of the immune system [25, 26]. In fact, HCV can infect and actively replicate in immune cells, thus directly affecting T cell functions [18].

Our study revealed that HCVne entry process started with the attachment of HCVne particles at the cell surface, which was followed by internalization and this event triggered different MAPK signaling pathways in human T cells. As shown, HCVne internalization in T cells induced p38 MAPK activation at 30 min post-internalization—a phenomenon which mimics that observed with other viral pathogens that activate MAPK pathways during viral entry [69, 70]. In cells of hepatic origin, endocytosis of HCVne is a clathrin-mediated process that requires p38 activation [25]. This activation is also observed in T cells that are incubated with HCVne and shows a similar kinetic pattern, thereby indicating an important role in internalization process. However, we do not have enough information, at present, to define the exact mechanism by which HCVne particles are able to activate the p38 MAPK pathway. Even so, the rapid nature of the initial activation suggests that it occurs early during entry, perhaps due to interactions between HCVne particles and cell surface molecules. Numerous reports have described the ability of the core protein to interact with T cell receptors and regulate T lymphocyte responses that are critical to viral clearance [29, 71, 72]. This possibility is further supported by the observation that heat-treated HCVne or control fractions did not induce phosphorylation of p38/MAPK.

Another interesting observation is that the transcription factors CREB and ATF-1 shared the same kinetic pattern with p38 activation, after HCVne internalization. These factors are known to be downstream targets of p38 signaling, and were previously reported to be phosphorylated via MK2 or MSK1/2 [73]. A parallel activation of $ERK_{1/2}$ was observed, which was sustained for up to 12 h. $ERK_{1/2}$ activation can also occur from a surface receptor or late endosomes [29, 74, 75]. Further investigation is needed to better clarify this phenomenon.

At the same time, the IL-2 gene showed a transcriptional activation between 2 and 12 h post-incubation, after binding and subsequent uptake of HCVne particles by T cells. This adds to previous studies, which proposed that the p38-ERK interaction in T cells may be of paramount importance for preventing inopportune IL-2 production and subsequent T cell activation and promoted the model that p38 acts as a gatekeeper [39]. It should be noted that p38 activity has differential outcomes, as far as IL-2 transcriptional activation is concerned, depending on the activation levels of ERK and JNK. Nevertheless, a parallel activation of p38 and $ERK_{1/2}$ results in activated effectors T cells and modulation of IL-2 transcription [76].

Immediate early gene *egr-1* showed transcriptional activation at early hours post-incubation of T cells with HCVne. *Egr-1* transcriptional activation has been reported after murine hepatitis virus entry and infection and correlates with the establishment of viral persistence [77]. Additionally, by comparing the kinetics of CREB and ATF-1 phosphorylation (Fig. 2C), along with *egr-1* mRNA induction (Fig. 4B), we can observe a similar pattern of activation, supporting the possibility that these two transcription factors are phosphorylated by p38 [78]. Phosphorylated CREB can also bind to the CRE elements of IL-2 promoter and activate IL-2 transcription [29, 79].

Moreover, not only *egr-1,* but also *c-fos* immediate early gene was activated as well. It is known that *c-fos* heterodimerizes with c-jun to compose AP-1, another key component for activation of the IL-2 promoter [80]. Especially for c-fos, we observed that the protein is stable in the nucleus of Hut-78 cells for several hours post-incubation with HCVne, probably due to the observed sustained ERK 1/2 activation that stabilizes the protein and results in continuous IL-2 expression. A similar phenomenon was previously described from our laboratory in cells of hepatic origin after HCVne internalization [25]. Since it has been shown that increased intracellular calcium levels are able to account for *c-fos* and *egr-1* gene expression [81], we could speculate that internalization of HCVne may trigger this pathway.

Another interesting observation is that when we used SB203580, a specific p38 inhibitor, IL-2 transcriptional activity reached mock basal levels, indicating the

importance of p38 in this activation cascade. In contrast, when MEKs were blocked by UO126, IL-2 transcriptional activity was not affected. These data clearly indicated that in the context of HCVne uptake, p38 is a key regulatory component of IL-2 gene expression, while the activation of MAPK/ERKs alone was not sufficient to induce IL-2 production. Taken together, these observations further support the idea that the direct or indirect interaction of p38 with ERK regulates important functions in T cells, such as cytokine expression [39]. In accordance with the kinetic pattern of p38-induced IL-2 transcriptional activation observed in T cells challenged with HCVne, we also observed a parallel secretion of IL-2 cytokine.

In human T cells, p38 seems to preferentially modulate IFN-γ expression that is induced either by IL-12/CD28 or by TCR/CD28 stimulation, suggesting that different co-stimulatory molecules might induce IFN-γ expression through the p38 pathway [53]. In our experiments, IFN-γ activation was specifically upregulated between 4 and 18 h in T cells after incubation with HCVne particles, indicating proinflammatory cytokine production. It is well known that IL-2 can also enhance IFN-γ expression in T cells, thus promoting a possible Th1-like response [29, 82, 83]. HCV is reported to affect IFN-γ signaling when infecting Molt-4 T cells [18], providing evidence for a TCR/CD28 type of stimulation. Fourniller et al. [84] reported that HCV infection can induce IFN-γ production and lead to CD8+ T cells enrichment in culture. Moreover, a significant increase in intracellular IFN-γ expression was reported in PBMCs that have been exposed to different HCV strains (MacParland et al. P-28 abstract presentation in the 16th International Symposium on Hepatitis C Virus and Related Viruses, Nice, 2009). A similar phenomenon was previously reported, describing cytokine induction by the HBV capsid [85].

A comparison between IL-2 and IFN-γ kinetics indicates that IL-2 activation precedes IFN-γ transcription. This expression profile agrees with the model of T cell exhaustion, recently proposed for HCV persistence [12, 86]. It has already been reported that during chronic viral infections, T cell exhaustion often correlates with poor control of viral replication [29, 87–89].

Finally, our observation that HCVne particles can bind and be internalized in T cell lines, as well as primary human PBMCs and intrahepatic mononuclear cells making them capable of stimulating IL-2 transcriptional activation, comes in accordance with the notion that recombinant in vitro-assembled HCV core protein is able to induce a strong specific immunity [90] and can provide evidence for additional features of particulate HCV core protein important in HCV life cycle and pathogenesis of HCV infection.

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